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Mitotic Pattern in the Chick Pronephric Duct

by JANE OVERTON[1]

From the Hull Zoological Laboratory, University of Chicago

WITH ONE PLATE

INTRODUCTION

BY means of a variety of ingenious experiments it has been shown that the pronephric duct originates anteriorly and grows caudally to join the cloaca (for review, see Burns, 1955). It has been concluded on the basis of histological study (Lillie, 1919), and from operative experiments in which the pronephric duct path was blocked by grafts (Waddington, 1938), that in the chick the pronephric duct extends posteriorly by proliferation of its own cells. In the present study, the proliferative pattern in the outgrowing duct of the chick has been examined in normal and in colchicine-treated embryos. Results indicate that proliferation is particularly marked towards the posterior duct tip. It was also noted that tensions of the blastoderm (see Spratt, 1956) which are disrupted by colchicine treatment appear to play some part in maintenance of normal diameter and posterior elongation of the duct.

MATERIALS AND METHODS

In this work attention was largely confined to chick embryos at stage 14 (Hamilton & Hamburger, 1951), though in a few cases stages 13 to 15 were included. The material consisted of both normal and colchicine-treated embryos. The normal group included embryos fixed at 21 to 24 somites. In the second group embryos of 20 to 22 somites were treated with colchicine by dropping 1·5 c.c. of colchicine 1:10^4 made up in Tyrode on top of the blastoderm through a window in the shell, resealing the egg, and incubating it 4 hours. With this treatment three-quarters or more of the embryos survived 4 hours as indicated by heart-beat. All were preserved. This group was at a developmental stage comparable to that of the first group despite the longer incubation period, since some cooling occurred during the treatment.

Counts were made of resting and dividing cells in both groups of colchicine-treated embryos. Embryos were sectioned at 10 μ and stained in Harris haematoxylin. Counts were made with an oil-immersion objective. They included

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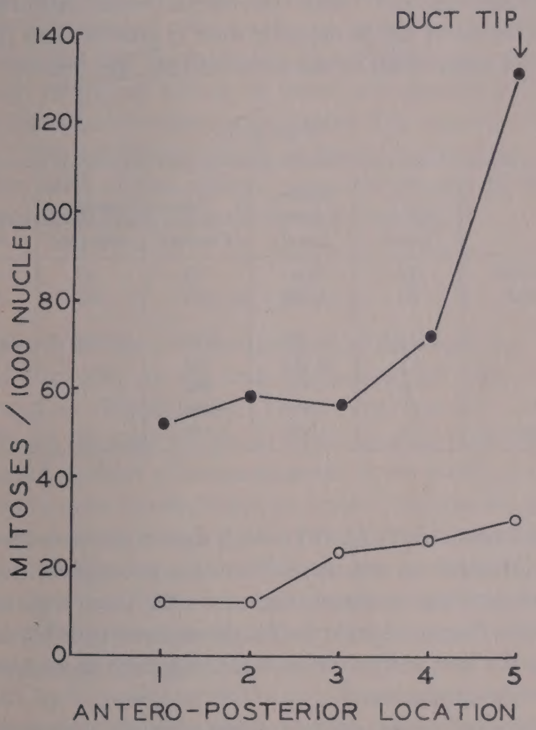
stages from late prophase to telophase in normal embryos and late prophase to metaphase in colchicine-treated embryos. In the pronephric duct counts were made from the posterior tip to the first branching tubule. The number of mitoses in anterior regions where new tubules join the duct appears to be high, possibly due to inductive activity in this region (see Bellairs, 1955). This anterior level was excluded from consideration. Mitoses were counted in every section of the duct, those occurring in more than one section being recorded only once. The number of resting nuclei in each section was also counted. The mitotic frequency was expressed as mitoses per 1,000 resting nuclei and was calculated for successive units of ten sections, or 100 μ lengths of the duct. Since ducts were of different lengths in different embryos, or even on right and left sides of the same embryo, the posterior tip of the duct was always taken as the point of reference in averaging counts from different cases.

Mitotic counts were made in somites and neural tube for comparative purposes at the most posterior level, which included both pronephric duct cords. The counts were made in six alternate sections, in two of which resting nuclei were also counted. The number of resting nuclei was then tripled in estimating mitotic frequency. The total numbers of resting cells in somite or spinal cord which appear in Table 1 are thus three times the number actually counted. In these counts the number of mitoses was usually recorded accurately but the number of resting nuclei occasionally differed by as much as 3 to 5 per cent. in successive counts of the same section. Because of differences in the counting method of mitotic figures in the duct and other regions, duct counts were adjusted for comparative purposes to take into account the section thickness, and it was assumed that the mitotic figure measured 4 μ (see Abercrombie, 1946). These adjusted counts were used in calculating the indices which appear in Table 1. This method can, of course, give only a rough measure of mitotic frequency. However, it seems adequate for comparing differences between various embryonic regions, and the mitotic indices obtained here are, in general, consistent with those reported by other workers (Schultz, 1922; Derrick, 1937; Bellairs, 1955).

RESULTS

Spatial mitotic pattern

Counts made throughout the duct and mitotic indices calculated for 100-μ duct lengths suggest that to a large extent the duct elongates through terminal proliferation. As indicated in Text-fig. 1 (open circles), average values based on counts in 24 ducts of normal embryos support this opinion. This trend was evident in many but not all individual cases. Such individual variation could be explained by the fact that the cell count for a given antero-posterior location might be as low as 50. When counts were repeated in embryos which had been exposed to colchicine for 4 hours, a marked and consistent posterior increase occurred. Averages for mitotic indices in 16 cases are plotted in Text-fig. 1 (solid circles). Since there is an over-all increase in the mitotic index in colchicine-treated embryos, the differences between anterior and posterior regions is accentuated. A rough correspondence between the two curves may be noted, since anteriorly the mitotic index increases from about 15 to 60, or four times, and at the tip the increase is from 30 to 135, again approximately four times. A very close correspondence would not be expected since, as will be described below, colchicine treatment produces some morphological distortion of the embryo. The data indicate that a definite spatial pattern occurs in mitotic frequency and, presumably, in proliferative activity. Thus proliferation would be highest towards the elongating tip.



TEXT-FIG. 1. Spatial distribution of mitotic activity in the elongating pronephric duct. Open circles, normal embryos; solid circles, colchicine-treated embryos. For explanation, see text.

This general antero-posterior pattern of mitotic activity is not confined to the duct alone, but is evident on inspection in colchicine-treated embryos throughout the axial mesoderm (see Derrick, 1937) and in the neural tube.

Relative mitotic frequency

Although mitotic activity is high at the duct tip compared to more cephalic levels, it is not higher than that in other embryonic regions at the same antero-posterior level. The situation is summarized in Table 1, which compares nuclear and mitotic counts in the most posterior 100-μ length of the duct with those in somite and spinal cord. In both somite and spinal cord the mitotic index in colchicine-treated embryos is 3·7 times the normal value. Although the increase produced by colchicine in the pronephric duct is greater than this, if counts in two unusually high cases, both in the same animal, are deleted, then the value for the pronephric duct is 3·8 times (Table 1, figures in parentheses), suggesting that there is no difference in this regard between pronephric duct, somite, and spinal cord. A comparison of mitotic indices in the three regions indicates that mitotic frequency in the pronephric duct is the same or possibly somewhat lower than that in adjacent mesoderm, while values for both mesodermal regions are lower than that for the spinal cord.

TABLE 1

Region	*No. of cases*	*Resting nuclei (total)*	*Mitoses (total)*		*Mitotic index*	*Indices colchicine/normal*
			Counted	*Adjusted*		
Pronephric duct (normal)	24	1,043	32	43	41·2	. .
Pronephric duct (colchicine)	16	1,257	173	242	192·2	4·7
	(14)*	(1,105)	(123)	(172)	(155·6)	(3·8)
Somite (normal)	12	6,261	328	. .	52·4	. .
Somite (colchicine)	8	4,932	948	. .	192·2	3·7
Spinal cord (normal)	12	9,906	929	. .	93·8	. .
Spinal cord (colchicine)	8	6,852	2,359	. .	344·2	3·7

* See text.

Counts in individual cases showed some over-all variation, most readily explained as due to differences in cooling. Since counts in various regions are compared here between whole groups, this variation is not apparent.

Colchicine distortions

In the colchicine-treated embryo both resting and dividing cells tend to become rounded. Such changes in cell shape are associated with a marked shrinkage of the *area vasculosa* radially and an antero-posterior contraction of the embryo proper, particularly in the trunk region, combined with abolition of normal elasticity of the blastoderm. A detailed study of these colchicine effects is described elsewhere (Overton, 1958). After colchicine treatment the pronephric duct is shortened and considerably thickened (Plate, fig. A). Effects of the antero-posterior contraction are particularly evident in resulting convolutions of the neural tube (Plate, fig. B). The consequent increase in cell count may be noted from data in Table 1. The greatest increase occurs in the pronephric duct where the cell count is approximately doubled. The increase is more moderate in the somite and least conspicuous in the spinal cord.

The shortening of the duct appears to a large extent to be a reflection of the rounding up of cells which are normally elongated in the direction of the antero-posterior axis. It may be contrasted, for example, with effects in the spinal cord where cells tend to be elongate at right angles to the antero-posterior axis. The duct tip, then, is composed of cells which are normally markedly elongate in the direction in which the duct is extending, a condition suggestive of migration. Attempts to study migratory activity of these cells directly by culturing sections of the trunk in plasma clots were unsuccessful. The increase in diameter of the pronephric duct with colchicine treatment must also be in part the result of distortions in other parts of the embryo, since shortening of the trunk may be sufficiently extensive to bend the notochord, for example.

DISCUSSION

Backward growth of the pronephric duct in the chick has been ascribed to proliferation of duct cells on the basis of histological study (Lillie, 1919) and surgical interruption (Waddington, 1938). The 'growth energy' of cells in terminal portions of the duct was noted by Gruenwald (1942) in cases where the duct was damaged a short distance anterior to its growing end and the part caudal to the injury grew normally and persisted. The mitotic pattern in the duct described here is consistent with the results of these operative experiments in suggesting that normally elongation of the duct is due largely to activity of cells at the posterior duct tip. Since a marked antero-posterior gradient in mitotic frequency occurs throughout the axial region, the proliferative pattern of the duct corresponds, at least roughly, to that throughout the trunk, and the mitotic index in the duct tip is similar to that in neighbouring mesoderm.

Although there is an early report of terminal mitoses in the outgrowing pronephric duct of *Triton alpestris* (Mollier, 1890), this spatial pattern is not characteristic of a number of *Ambystoma* species (Overton, 1959) in which mitoses appear to be rather uniformly distributed along the length of the duct. The amphibian duct seems to extend posteriorly as a result of the activity of cells throughout its length, while in the chick the actively growing region is more localized.

In colchicine-treated chick embryos there is a conspicuous shortening of the duct and an increase in its diameter. This distortion is most marked in the duct tip, where it results to a large extent from changes in shape of duct cells which become rounded. Duct cells thus tend to be elongate, particularly towards the tip, suggesting that cells here are involved in migration as well as in multiplication. Changes in cell shape in other regions associated with loss of elasticity of the blastoderm and with antero-posterior shortening of the embryo also affect the arrangement of duct cells indirectly. These distortions emphasize the role of normal tensions of the blastoderm in maintaining the typical diameter and extent of the pronephric duct.

SUMMARY

1. Studies carried out on the stage 14 chick embryo indicate that in normal and in colchicine-treated embryos mitotic frequency is greatest towards the posterior tip of the elongating pronephric duct.

2. Although the mitotic frequency in the duct tip is high compared to that in the duct at more cephalic levels, it is similar to that in other mesodermal regions at the same level.

3. Distortions of the embryo which occur with colchicine treatment suggest that migratory activity is prominent towards the duct tip, and emphasize the dependence of normal morphology of the duct on tensions in the blastoderm.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

FIG. A. Cochicine-treated chick embryo; cross-section. *ca.* ×475.

FIG. B. Cochicine-treated chick embryo; fixed preparation. *ca.* ×37.

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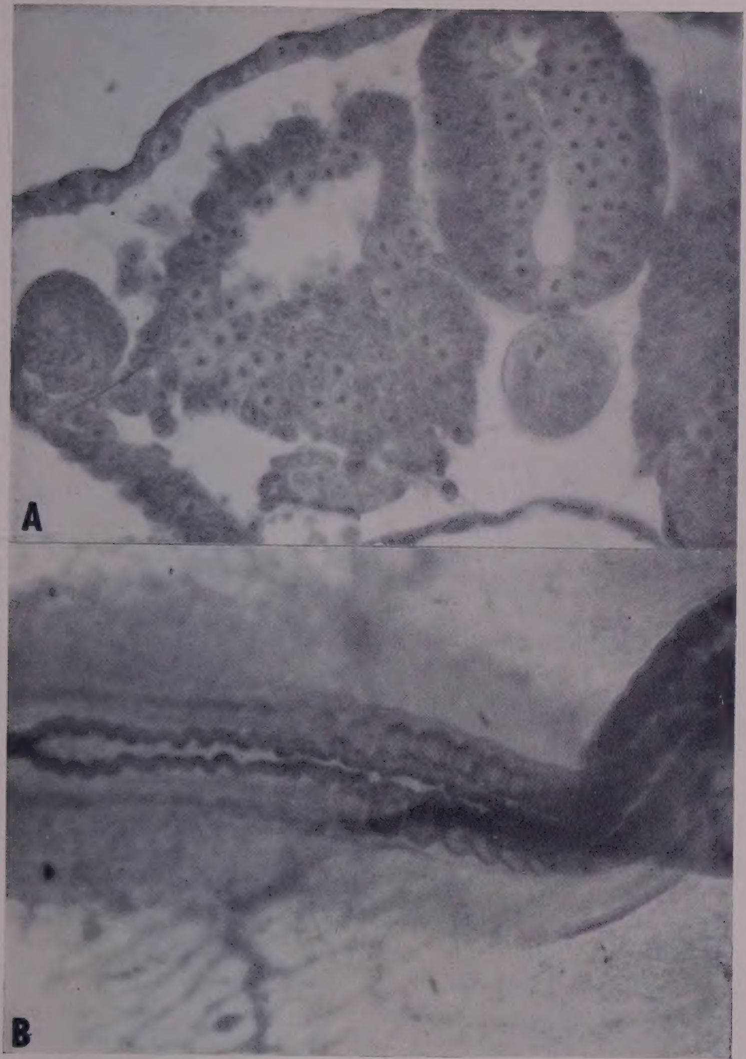

J. OVERTON

Tail Regeneration in the Geckonid Lizard, *Sphaerodactylus*

by ARTHUR HUGHES[1] *and* DENIS NEW[2]

From the Department of Zoology, University College of the West Indies, and the Anatomy School, Cambridge

WITH ONE PLATE

INTRODUCTION

In the *Histoire de l'Academie Royale des Sciences* for 1686 (vol. ii, p. 7, published 1733) under the heading of 'Diverses Observations Anatomiques' is an account of the researches of three savants, Thevenot, Du Verney, and Perrault, on regeneration of the tail in the European Green Lizard. Perrault found that in the new tail, instead of vertebrae, 'il n'y avoit qu'un cartilage de la grosseur d'une grosse épingle'. It was not until nearly a century and a half later that this basic observation was extended when Dugès (1829) described this element of the regenerate as 'un cartilage d'une seule pièce, blanc, flexible, fistuleux, et rempli d'un prolongement du cordon ou faisceau nerveux rachidien'. Not before the main framework of microscopical anatomy had been built in succeeding generations was the exact nature of this nervous component determined. Gegenbaur (1862) realized that although it was a continuation of the spinal cord, the full medullary structure was lacking. In the next year Heinrich Müller (1863) described how it was made up of an 'Epithel umgebener Kanal' covered by a layer of longitudinal nerve fibres, between which and the inner epithelium were cellular elements, 'nicht mit Sicherheit als Nervenzellen anzusprechen'. On the other hand, Fraisse (1885) illustrated neuroblasts within the neural tube in tail regenerates of *Lacerta ocellata* and of the slow worm. Since the work of Hooker (1912), however, it has generally been agreed that this structure is a continuation of the ependymal epithelium, infiltrated with some glial elements, and covered with fibres from the white matter.

Fraisse's treatise was a general monograph on regeneration, mainly in Amphibia and Reptiles. In the lizard tail he described and illustrated the arrangement of the muscle bundles and the development of the scales. He saw that the

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regenerate is innervated from spinal nerves near the junction of new and old tissue. His longitudinal section through a regenerating tail of *L. agilis* shows branches of the last pair of spinal nerves entering the new growth and running between the cartilaginous tube and the annular zone of muscle bundles. This picture of the nervous system of the regenerate was not extended for nearly thirty years. In 1912 Hooker described the descent of branches of two pairs of spinal nerves into the new tail, while Terni (1920) showed that in *Gongylus ocellatus* three pairs of spinal nerves send branches into the regenerate. In a further paper, Terni (1922) described how fibres of the para-aortic sympathetic trunks grow into the new tissue, but are not accompanied by autonomic neurones.

Comparatively little has been written on the microscopical features of the early stages of regeneration and the histogenesis of the various tissues. Fraisse (1885) considered that each was derived from cells which originated from the corresponding tissue in the stump. For instance, he believed that special cells, the 'Muskelkörperchen', migrated out from the original musculature to form the new muscle fibres. White (1915, 1925), however, describes how beneath the epidermis which grows over the broken surface of the tail there accumulates a 'mass of undifferentiated cells mixed with pigment cells' which constitutes a blastema, within which the new tissues differentiate. The views of Quattrini (1954) on this question recall those of Fraisse. He considers that cells emerge from the intermuscular septa of the stump and form the muscles of the regenerate, and that the periosteum of the broken vertebra contributes formative cells for the new skeletal tube.

The present paper is concerned with tail regeneration in the small West Indian Geckonid *Sphaerodactylus*, chiefly with regard to the early phases of the process. Some points were compared with similar events in *Aristelliger*, the 'Croaking Lizard'. Some preliminary experiments on the effect of the treatment of the regenerate with nitrogen mustard are also described. The purpose of these experiments was to compare the effect of this substance on the regenerating tail with its action on the limb-buds of *Xenopus* tadpoles (Hughes & Tschumi, 1958), in the first instance with regard to the nervous system of the regenerate.

MATERIAL AND METHODS

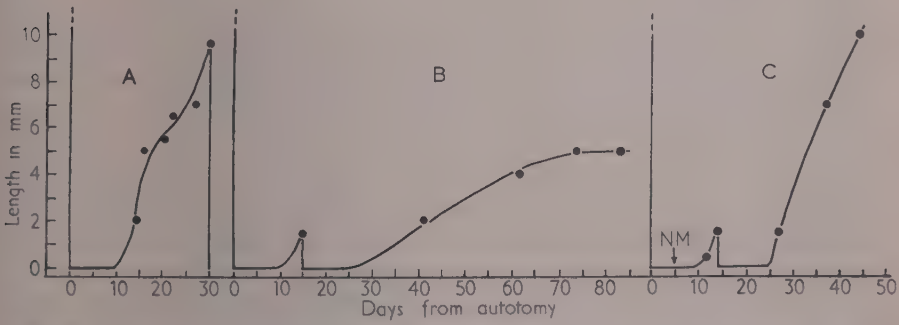
Individuals of the smaller species of *Sphaerodactylus* (*goniorhynchus* or *argus*) were collected at various places in Jamaica, mostly within a few miles of the University College of the West Indies, at Mona, St. Andrew. The majority were found beneath the trash under coco-nut palms. The length of the animals used varied between 30 and 40 mm.

The animals were kept in the laboratory in small museum jars covered by wire gauze and kept on a diet of larvae and adults of local species of *Drosophila*. *Sphaerodactylus* readily sheds portions of tail about 10 mm. in length in response to gentle traction on the extremity; approximately this length of tail was removed in all the experiments here described. Seven to ten mm. of tail remained between cloaca and the plane of autotomy. Measurements were made of the growth of the regenerate, samples of which were again 'plucked' at various stages. The animals were kept at room temperature varying between 25° and 30° C. The material was fixed in Bouin's fluid at full strength for 24 hours, followed by dehydration and infiltration with wax at 60° C. Sections were cut in an air-conditioned room at 21° C. The sections were stained either with Delafield's haematoxylin and eosin, or were silvered by either Holmes's or Bodian's methods. In treating the regenerating tails with nitrogen mustard it was found possible to immobilize the animal by wrapping cotton wool around the trunk and limbs. Solutions of methyl-bis (βchlorethyl) amine hydrochloride at various concentrations were applied for periods of up to 5 minutes. The treated regenerates were plucked after various intervals and cut into serial sections, which were impregnated with silver.

RESULTS

The rate of regeneration in Sphaerodactylus *and other lizards*

There are three phases in the growth of the regenerating tail. First there is an interval during which the blastema is being formed and in which little or no growth is visible externally. Then follows a period of rapid elongation, the rate of which finally decreases as the regenerate approaches its full length. The first two of these phases in the normal growth of a first regenerate in *Sphaerodactylus* are shown in Text-fig. 1A. Repeated plucking of the regenerate results in a much slower rate of growth (Text-fig. 1B).



TEXT-FIG. 1. Length of individual tail regenerates in *Sphaerodactylus*. Vertical lines indicate autotomies, at the first of which (0 in abscissa) 10–12 mm. of original tail was shed. A, growth of first regenerate. B, growth of second and third regenerates. C, growth of second and third regenerates as in B except for treatment with 1 : 500 nitrogen mustard for 10 minutes (NM) during second period.

In the experiments with nitrogen mustard, the regenerating surface was treated with the substance at various times after plucking of the original tail. When the nitrogen mustard was applied during the first phase of regeneration, growth was either halted or much slowed down. If, however, after such a period of dormancy the last segment of the tail was itself plucked, then normal regeneration ensued. In the two experiments of this kind the rate of growth was the highest ever observed (Text-fig. 1c).

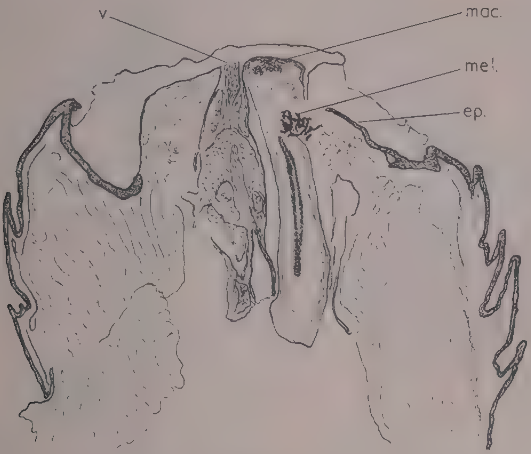
The maximum rate of growth of a normal first regenerate in *Sphaerodactylus* was about 0·47 mm. per day. In *Anolis carolinensis*, Kamrin & Singer (1955) measured rates of growth during the second period of 0·4 mm. per day. Gosse (1851) observed in *Thecodactylus* (*Aristelliger*) a maximum of about 0·7 mm. per day; in *Hemidactylus flaviviridis* a rate of 1·12 mm. per day can be deduced from Woodland's (1920) drawings. In *Lacerta* there are records of still more rapid elongation, namely 1·36 mm. per day (Hooker, 1912) and 2·0 mm. per day (Holfert, 1869, quoted by Fraisse, 1885, p. 34). The seventeenth-century observations of Thevenot and Perrault (see Introduction) also suggest figures of this order for European lizards.

Histological observations

The microscopical structure of the regenerating tail during the earliest phases of the process will be described from a series of specimens taken at various times and cut into longitudinal sections.

Two days after the tail has been shed the soft tissues of the healing stump are covered by a crust of necrotic cellular material through which the broken surface of the autotomized vertebra projects. Beneath this covering, cellular reactions to the injury are already apparent. At the margins of the broken surface blood-vessels are dilated, and from them lymphocytic elements are spreading over the inner surface of the protective crust. Within the neural canal of the broken vertebra, the spinal cord has withdrawn some 60 or 70 μ from the surface. The open end of the central canal has begun to dilate, and beyond it cells from various sources are accumulating. Some are microglial elements of the cord; others are lymphocytes from dilated vessels of the meninges. From these membranes come also melanocytes, which are beginning to collect in a compact group on the dorsal surface of the cord. There are occasional mitotic figures among these cells within the neural canal.

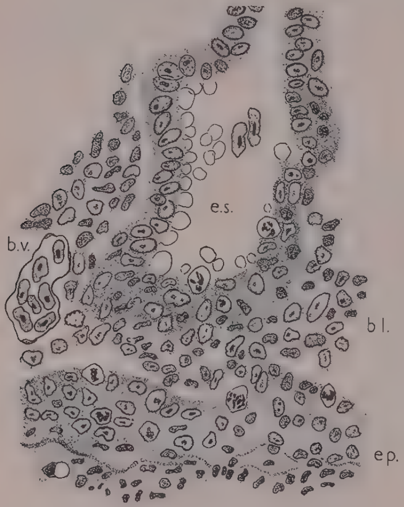
A day later (Text-fig. 2), the cord has retreated still farther within the neural canal, the open end of which is occupied by a mass of cellular material, now much denser than on the previous day. There is a tight knot of melanocytes on the dorsal surface of the cord at its hinder limit. Beneath the external crust, a dense layer of macrophages is loaded with cellular debris. Round the edge of the wound, the epidermis has begun to proliferate, and in a longitudinal section a tongue of ectodermal cells is seen on each side turning sharply inwards and growing over the outer surface of the intact tail musculature and perivertebral fat and beneath the necrotic tissue to the outside. The inner edge of this circle of ectoderm advancing centripetally meets in the centre on the 4th day, when a thin layer of epithelium covers the neural canal and the plug of new tissue within it. On the 5th day, this new epidermis becomes everywhere much thicker, and within it the distinction between an inner germinative layer and an outer *stratum corneum* becomes clear. The outer edge of the new epidermis joins the old in an even thicker rim which gradually extends more deeply into the zone of regeneration. The new epidermis remains much thicker than the old until the time when it undergoes differentiation into scales.



TEXT-FIG. 2. Vertical longitudinal section through stump of tail of *Sphaerodactylus*, 3 days from autotomy. *ep.*, regenerating epidermis; *mac.*, layer of macrophages over open end of neural canal; *mel.*, knot of melanocytes at broken face of spinal cord; *v.*, autotomized vertebra. × 32.

However, the most striking new development at this stage is the outgrowth of the ependymal epithelium into a flask-shaped sac, comparable to that which has been observed in the regeneration of the cord in the tail of a Urodele (Duesberg, 1925). In *Sphaerodactylus* the sac extends axially outwards towards the germinative layer of the epidermis on the 4th day. At the base its wall consists of a single layer of columnar epithelium. Towards the tip it is surrounded by a dense crowd of new cells between the inner members of which and the ependymal epithelium no clear distinction can be drawn. Within the wall of the ependymal sac mitotic figures are far commoner than at any other site. The whole appearance suggests that this sac is an active centre of proliferation, and that at this stage it is the main site of the production of new cells for the whole zone of regeneration (Text-fig. 3). Already at 5 days the cells constitute a dense blastema in the form of an inverted bowl, the edge of which approaches the outer zone of the tail musculature. The individual cells of the blastema are remarkable for their variety in appearance in that their nuclei differ both in size and in the density of their contents. Over the inner surface of the blastema, melanocytes are migrating outwards from the tight group of earlier stages on the dorsal surface of the nerve-cord.

From the inner surface of the cells of the ependymal sac at 5 days, vesicles bulge into the lumen of the sac (Text-fig. 3). These are apparently a sign that cerebro-spinal fluid is being secreted by the epithelium. Occasionally blood-corpuscles are seen within the lumen.

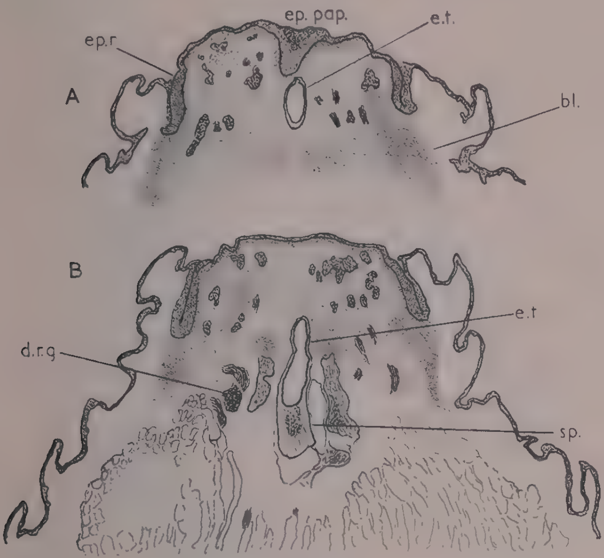


TEXT-FIG. 3. Part of median longitudinal section through regenerating tail area of *Sphaerodactylus* 5 days after autotomy showing ependymal sac (*e.s.*) close to regenerated epidermis (*ep.*), and the origin of cells of the blastema (*bl.*); *b.v.*, blood-vessel. × 333.

On the 6th day the influence of the ependymal sac, now an elongated tube, is apparent in another respect. At the centre of the new epidermis is an inward prolongation in the form of a papilla, pointing towards the tip of the sac (Text-fig. 4). The two structures are not in actual contact, but are separated by a few cells of the surrounding blastema. It may be that the ependymal sac at this stage stimulates a zone of the epidermis lying nearest to it to a maximal rate of proliferation. Melanocytes have by now entered the new epidermis and are particularly numerous within the central papilla.

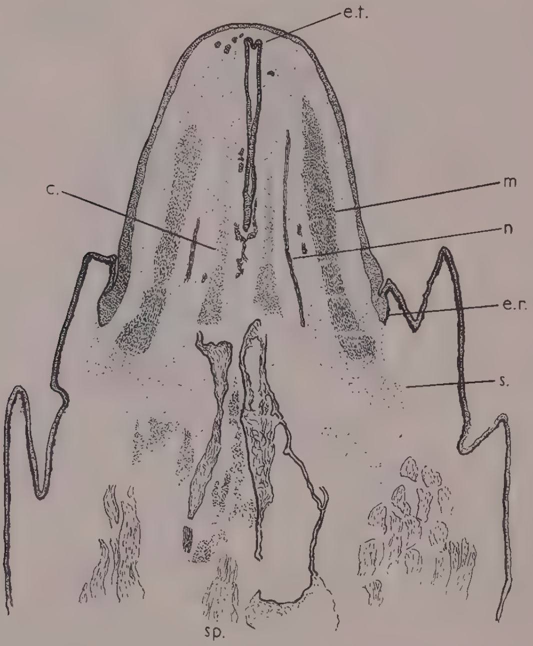
The regenerative blastema is now nearly 0·5 mm. thick at its axis. Inwards it presents a deepening concavity. The outer surface is limited by the deep rim of epidermis at the junction of new and old ectoderm. Below this rim is a specially dense ring of blastema, the *Anlage* of the musculature of the regenerate. Within the remainder of the blastema, two sets of structures are apparent: a plexus of irregularly dilated blood-vessels, especially concentrated near the epidermis, and the growing longitudinal branches of the spinal nerves, which will be described in detail below.

Within the next 5 days signs of cellular differentiation within the blastema become apparent as the regenerate begins to grow in length. The dense ring of blastema from which the musculature is formed becomes a hollow cone, and its constituent cells differentiate into elongated myoblasts. They become sharply distinct from the cells of the surrounding blastema. Mitoses are more common among the myoblasts than in the blastema generally; the axes of the mitotic figures of the myoblasts coincide with the general direction of growth (Plate, fig. B). They increase mainly by accretion; at the tip are the youngest myoblasts, hardly distinguishable from the cells of the surrounding blastema. Differentiation is most advanced at the base of the cone, and here the future segmentation of the muscle bands is already foreshadowed. These bands, when seen in transverse section, vary in number between ten and thirteen. In *Sphaerodactylus*, twelve is the usual number. At 11 days (Text-fig. 5) a wide space still lies between the myoblastic cone and the original musculature; it is occupied by a loose mesenchyme which is heavily infiltrated with lymphocytes, as is the whole zone of junction between new tissue and old. The myogenic region of the regenerate is derived from blastemal cells, and is independent of the original musculature of the tail. The latter must however exert an influence on the division of the cone of myoblasts into muscle bands, for each new band becomes a continuation of an old one.



TEXT-FIG. 4. Part of horizontal section through regenerating tail area of *Sphaerodactylus* 6 days after autotomy. (A) Near tip of ependymal tube, and (B) at its base. *bl.*, blastema, containing nerves and blood-vessels and bounded by denser myogenic ring; *d.r.g.*, dorsal root ganglion; *e.t.*, ependymal tube; *ep. pap.*, epidermal papilla; *ep. r.*, epidermal rim; *sp.*, spinal cord. ×42.

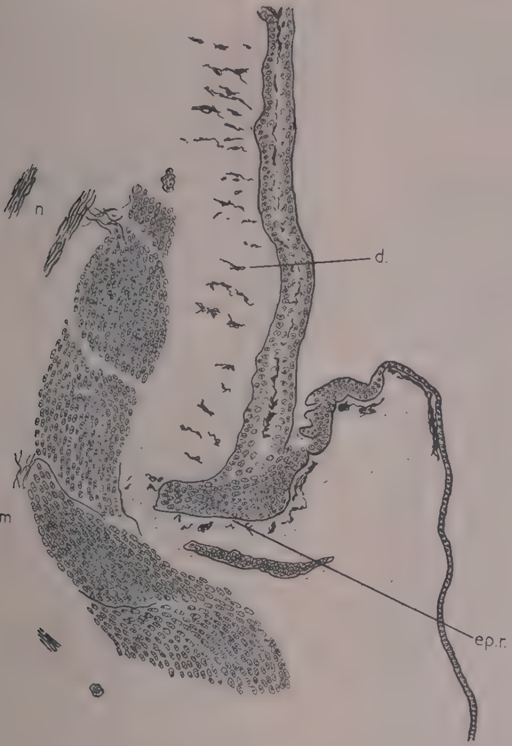
The ependymal sac, extended at 11 days into a tube nearly a millimetre in length, is surrounded by a further condensation of mesenchyme, consisting also of elongated cells; here, however, their axes are all in radial directions. These cells are the chondroblasts of the cartilaginous tube. By this stage there has differentiated a coarse plexus of blood-vessels surrounding the ependymal sac. The epidermis of the regenerate has the form of a thick hollow cone, the base of which is the peripheral rim of earlier stages. In this region, the outer surfaces of new and old epithelium join at the base of a deep annular fissure. Within the *stratum germinativum* mitotic figures are common everywhere, but they are especially prominent within the massive basal rim. Here melanoblasts are most frequent, both in the future dermal layer of the regenerate and also within the epidermis, into which pigment cells can be seen to migrate.



TEXT-FIG. 5. Vertical longitudinal section through 1-mm. tail regenerate of *Sphaerodactylus*, 11 days from autotomy. *c.*, developing cartilaginous tube; *e.t.*, ependymal tube; *e.r.*, epidermal rim; *m.*, myogenic band; *n.*, longitudinal nerve; *s.*, space between new and old muscle infiltrated with lymphocytes; *sp.*, spinal cord. ×43.

At 15 days (length of regenerate 1·5 mm.) the chondroblasts of the skeletal tube are beginning to lay down their intercellular matrix. The tube remains open at the tip for some days, where the vascular network at the tip of the regenerate joins with the peri-ependymal plexus. Within the myoblastic bands segmentation now extends nearly to their apices. Melanoblasts in both dermis and epithelium reach to the tip of the regenerate (Text-fig. 6).

In succeeding days the inner surface of the epithelium becomes uneven, and these irregularities develop into a series of internal ridges which in longitudinal section recall the now well-known internal cristae of mitochondria. The dermis is thus moulded into a series of bays, each of which will be the core of a future dermal scale (Text-fig. 7). Within each bay is a large vascular space, and at the base of the epidermal cristae is a dense mesenchyme. The epidermal segmentation into scales does not correspond with that of the muscle segments, for the former are the more frequent.



TEXT-FIG. 6. Part of longitudinal section through base of 1·5-mm. tail regenerate of *Sphaerodactylus* 15 days from autotomy. (Same specimen as in Plate, fig. E.) *d.*, dermis with melanocytes; *ep. r.*, epidermal rim; *m.*, segmented muscle band with nerve fibres in intersegmental plane; *n.*, longitudinal nerves. ×121.

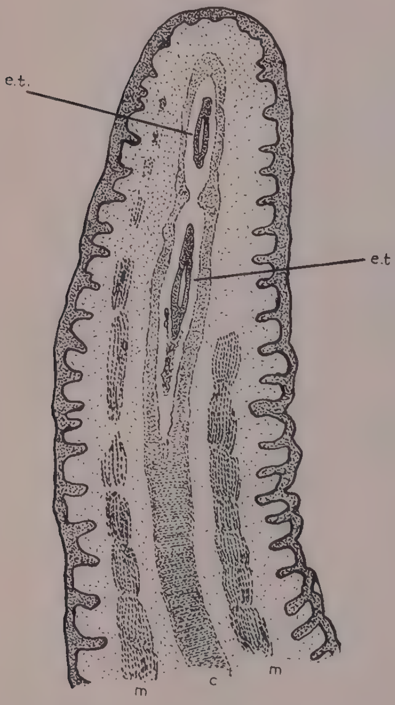
In regenerates between 5 and 6 mm. in length, the whole development of the skin is carried to a further stage. All but the youngest epidermal cristae become tilted so that they point away from the tip of the regenerate. In this way the imbricated arrangement of the scales of the regenerate is foreshadowed. Within the *stratum corneum* of the developing scales, dense lamellae of keratin are formed, but the whole outer surface of the regenerate is still covered by an even surface of cornified cells which fill the spaces between the young scales. At 7 mm. all that remains of this original outer layer is a hyaline cuticle. By this stage the outermost layer of the dermis is densely infiltrated by melanocytes, processes from which extend into the dermal core of the scale. As the scalation develops, the epidermis becomes much thinner than at earlier stages and then, as in the adult epidermis, no longer contains any pigment cells.

The differentiation of muscle fibres

In the 1-mm. regenerate (11–12 days) the muscle columns are distinct from the surrounding mesenchyme (Text-fig. 5). They are made up of elongated myoblasts, and towards the base of each column the division into muscle segments is already apparent. At 1·5 mm. (15 days; Text-fig. 6) the myoblasts in the older segments are becoming aligned into longitudinal rows. During the next 2 days each of these rows becomes a continuous strip of cytoplasm with a row of nuclei down the middle. Within the denser cytoplasm on either side of the nuclei can be seen longitudinal myofibrillae. The myoblasts have thus fused into multinucleate muscle fibres. At first they are densely packed together.

The regenerate is now elongating at its maximum rate; the greatest extension among the muscle segments is seen in that next but one to the original muscle of the tail. In a 5-mm. regenerate it is about 0·5 mm. in length, a threefold increase since the time when segmentation was first recognizable. The segment next to the original musculature of the tail is a specially short one, and serves to join the new band to the old.

As the muscle fibres elongate they become separated by clear spaces in which occasional fibroblast-like cells are seen. Pycnotic nuclei are so common at these sites as to suggest that cell degeneration is one factor by which the muscle fibres become separated from each other (Plate, fig. C). As the muscle fibre elongates, its nuclei increase from about twelve to twice this number. Mitotic figures gradually become less common, but do not cease before the muscle fibres become discrete.



TEXT-FIG. 7. Part of longitudinal section through 2·5-mm. tail regenerate of *Sphaerodactylus* 17 days from autotomy. The epidermal bays foreshadow the future scalation. *c.*, cartilaginous tube; *e.t.*, ependymal tube; *m.*, muscle band. ×50.

In *Aristelliger* mitosis within the muscle segments continues for a longer period after the first differentiation of fibres than in *Sphaerodactylus*, a distinction presumably correlated with the more rapid growth of the regenerate in the former. Most of the later mitotic figures remain orientated in the axial direction, but are generally to be found in cells lying either within the spaces between the muscle fibres or in contact with the surface of a fibre. Such cells show the rounding-off usual in the later stages of mitosis. We were not able to find an early prophase figure among nuclei within fibres to help us to decide whether the nuclei of the young muscle fibres can undergo division *in situ*. It seems most probable that in *Aristelliger* the muscle fibres grow in length by the intussusception of additional cellular elements derived from residual myoblasts within the muscle segments. Although mitotic figures are abundant among cells between the muscle fibres, there is no general increase in their density at these sites, while the nuclei within the muscle fibres steadily increase in number. It is further possible that new muscle fibres may also be formed to add to the number of those already differentiated in a similar way to that which Couteaux (1941) has described in mammalian myogenesis.

In *Sphaerodactylus* pycnotic nuclei are still seen within the muscle segments of regenerates in which multinuclear fibres have differentiated. Counts of the fibres at comparable levels in transverse sections of regenerates of different ages indicate that their total number decreases in the final stages of development. At this time the diameter of the fibres increases from about 60 μ to about three times this value. The spaces between the fibres are thus largely obliterated.

The first sign of cross-striation within the fibres is seen as a dotting of the myofibrils in the oldest segment of a 3-mm. regenerate. In a silvered preparation of a 7-mm. specimen, the *A*-disks are clearly distinguishable in the more proximal segments among fibres which have reached 100 μ in length. In mature Lacertilian muscle the nuclei remain at the axis of the fibre.

In the regenerating tail it is probable that the multinucleate muscle fibre is formed in the first place by the coalescence of myoblasts. The extensive literature on the histogenesis of muscle is generally divisible between descriptions of the fusion of myoblasts into muscle fibres (Godlewski, 1902; Asai, 1914) and, on the other hand, their origin from single myogenic cells (Iwanaga, 1925). It is probable that during the course of evolution there has arisen diversity in this respect. Franz (1915) finds that the muscle fibres of *Triton* are developed from uninuclear myoblasts, while in Arthropods the fibres have a multicellular origin. In the developing chick, both Meves (1909) and Weed (1937) agree that the single myoblast is the formative unit, but diverse opinions have been expressed on myogenesis in mammalian embryos (Baldwin, 1913; Assai, 1914). The regenerating lizard's tail provides extremely favourable material for the study of the formation of muscle fibres, and would repay investigation by more detailed methods.

The development of the nervous system of the regenerate

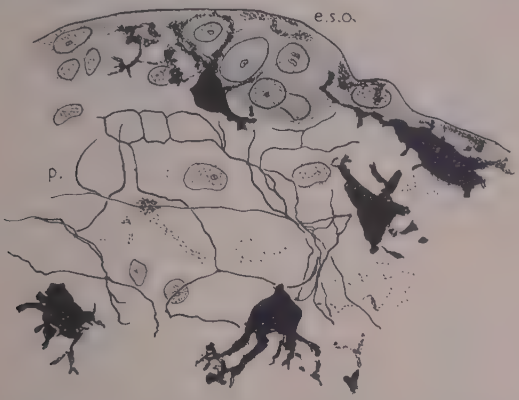
In *Sphaerodactylus*, as in *Gongylus* (Terni, 1920), branches of three pairs of spinal nerves run across each autotomy septum. When the tail is shed, fibres of the severed proximal ends of all these nerves resume growth and enter the zone of healing. In *Sphaerodactylus* these growing neurites are approaching the surface of the wound within 2 days of autotomy. When this becomes covered, on the 4th day, by a layer of new ectoderm, a plexus of fine nerve fibres can be traced among the cells of the inner surface of the new epithelium (Plate, fig. D). At their roots the growing fibres of the spinal nerves become swollen and at fixation are made irregular in outline. This feature is more marked in ventral roots than in dorsal roots; it is evident as early as the 4th day, and is still more marked on succeeding days (Plate, fig. A). As the ependymal sac develops, fibres of the white matter grow over the surface of this structure. These cord neurites begin their new growth on the 4th day; each is then dilated at the tip.

Within the dorsal root ganglia the perikarya show signs of the demands which the new growth of fibres imposes. Two days from autotomy all traces of Nissl substance vanish from the cytoplasm and the nucleus is then liable to an artefact of fixation in which the contents contract round the swollen nucleolus, leaving either an empty space around the inner margin of the cytoplasm or a number of large vacuoles (Plate, fig. A). Recovery of the perikarya is largely complete during the second week of regeneration. Distortion of the cell-body at fixation is absent in dorsal root ganglia not concerned with regeneration. A further feature to be seen within the dorsal root ganglia during regeneration is a heavy infiltration of lymphocytes from surrounding tissues during the 2nd and 3rd weeks after autotomy (Plate, fig. G).

As the apical blastema thickens there is a corresponding elongation of the nerve trunk. Each of the last three pairs of spinal nerves sends four backwardly directed branches into the regenerate. As Terni (1920) has shown in *Gongylus*, the last pair innervates that sector on either side of the regenerate which includes the median horizontal plane. Branches of the next pair traverse the adjacent sectors, while the mid-vertical plane is supplied by the most anterior of the three pairs of spinal nerves. The full number of 24 longitudinal nerves is seen at the base of the regenerate, but only about half of these reach the neighbourhood of the tip. These longitudinal nerves run in the loose mesenchyme between the cartilaginous tube and the inner surface of the myogenic zone. Smaller branches are given off which run between the developing muscle bands and towards the epidermis.

The nerves within the regenerate resemble those of an embryo in that they consist of fibres accompanied by immature Schwann cells, the distribution of which remains uniform in density during the continued elongation of the nerve (Plate, fig. I). Mitotic figures among the Schwann cells are very rarely seen, yet their total number is continually augmented, presumably from the blastema at the tip of the regenerate, which must be made up of cells of various presumptive potencies.

Branches of the longitudinal nerve trunks pass outwards between the muscle bands towards the surface of the regenerate. At 3 mm., fibres from these sensory nerves have everywhere reached the epidermis into which fibres have hitherto penetrated only at the tip. As soon as the future scalation is foreshadowed within the outer layers of the regenerate, a sensory nerve enters the dermis of each young scale and gives off a plexus of fine fibres which run towards the surface. In an 11-mm. regenerate, at the tip of each scale can be seen a developing epithelial sense-organ consisting of a cluster of cells thicker than the surrounding ectoderm, and beneath which the sensory plexus is especially prominent (Text-fig. 8). These tactile organs are completed by the acquisition of hair-like prolongations of the surface cuticle, forming organs of the type which Schmidt (1921) has described in *Calotes* and other lizards. These processes are readily seen in the shed cuticle; the earliest stage at which we have seen them in a regenerate was at 45 days.

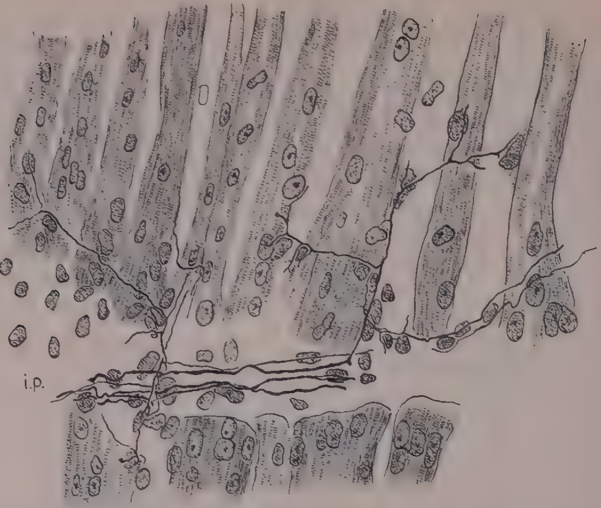


TEXT-FIG. 8. Part of section through scale of 11-mm. tail regenerate of *Sphaerodactylus* at 22 days showing epidermal sense organ (*e.s.o.*) at tip of scale. *p.*, dermal plexus of nerve fibres. ×864.

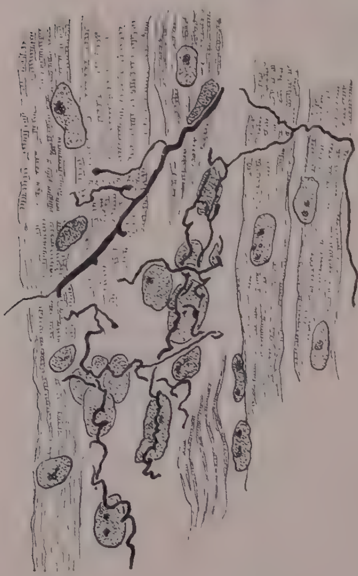
As soon as the segmentation of the muscle bands is detectable, and while their constituent cells are still in a myoblastic stage, single nerve fibres leave the longitudinal trunks and enter the myocommatal-like intersegmental planes. Such neurites will become the motor fibres of the regenerate. They first enter the base of the muscle bands at 15 days, the regenerates being 1·5 mm. long (Text-fig. 6; Plate, fig. E).

Gradually, more fibres enter the intersegmental planes accompanied by Schwann cells. In 4-mm. regenerates the whole length of each muscle band is segmented and has received motor fibres in all intersegmental planes.

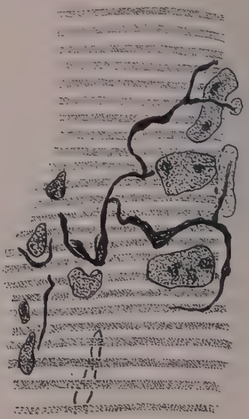
A further stage in the development of the innervation of the musculature is



TEXT-FIG. 9. Part of longitudinal section near tip of muscle band of 11-mm. tail regenerate of *Sphaerodactylus* at 22 days showing intersegmental plexus of motor fibres (*i.p.*) with Schwann cells spreading along muscle fibres. × 500.



TEXT-FIG. 10. Part of longitudinal section through oldest segment of muscle band of 11-mm. tail regenerate of *Sphaerodactylus* at 22 days. Motor fibres, accompanied by Schwann cells, have migrated along muscle fibres and are forming end-plates. × 1,280.



TEXT-FIG. 11. Mature motor end-plate within original musculature of tail of *Sphaerodactylus*. × 1,200.

reached by the migration of the motor fibres, accompanied by Schwann cells, to either side of the plane of entry and along the surface of the muscle fibres (Text-fig. 9). The motor fibres soon become contorted and twisted round the Schwann cells (Text-fig. 10). Together these two components gradually assume the form of the mature end-plates characteristic of Lacertilian muscle (Text-fig. 11) as described by Bremer (1882) and more recently by Wilkinson (1931). In an 11-mm. regenerate only in the most mature muscle segments has the motor innervation spread along the whole length of the muscle fibres.

In other vertebrates it is not generally agreed that the nuclei of motor end-plates are always derived from Schwann cells, though Tiegs's (1953, p. 125) review of this subject suggests that they are the most probable source of these elements. In the regeneration tail of the lizard, thanks to the entry of motor nerves and Schwann cells into the muscle segments from the ends of the muscle fibres, it seems clear that at least some end-plate nuclei come from Schwann cells, though the possibility cannot be excluded that fibroblasts of the inter-segmental planes may contribute thereto.

Development of function in the neuromuscular system of the regenerate

The activity shown on autotomy by an original tail or a mature regenerate, free of cortical control, is extremely violent. In *Aristelliger*, for instance, one sees circular waves of contraction propagated round the tail with the effect that the shed member rotates briskly on the ground for some minutes. In an immature regenerate movement is less evident, and may be no more than a faint unco-ordinated twitching of each muscle band. In *Sphaerodactylus* this is seen in a shed regenerate 7 mm. long, but one of 4 mm. remains immobile.

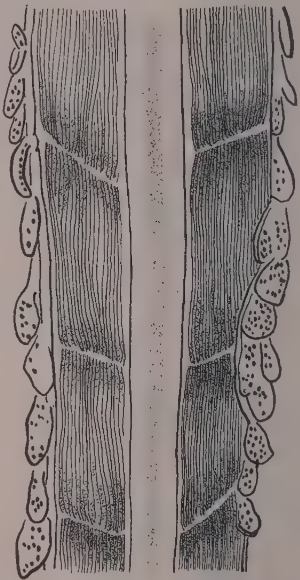
A further approach to this general question was made by the application of the diazonium method for the detection of esterases (Lewis, 1958) to developing regenerates of various ages. At autotomy the shed organ was plunged in chilled formalin and bisected along a median plane with a razor blade. Lewis's procedure was then followed, and the cut surface of the regenerate was finally examined for the appearance of the reddish-brown product which indicates the presence of an esterase and, in this connexion, presumably of cholinesterase (Text-fig. 12). In a 7-mm. regenerate (30 days), a strong reaction was evident between each of the older muscle segments, but in a 4-mm. regenerate, where no movement was seen on plucking, a faint coloration was only just detectable in the intersegmental planes. At this stage motor fibres have not spread beyond these sites, and their endings are still in an undifferentiated condition.

The effect of nitrogen mustard on tail regeneration

The experiments on the effect of nitrogen mustard on tail regeneration were divided into two groups. In the first the substance was applied 5 days after autotomy at a stage when the wound is covered by epithelium beneath which is a regenerative blastema in its earliest stages. Of eleven animals treated in this way, in two there was no growth externally visible in the 3rd week. In the remaining nine the regenerate began to grow after the usual 10–11 days after autotomy, but elongated at rates below the normal for first regenerates. In the second series of experiments a group of regenerates 4–5 mm. in length were treated with nitrogen mustard and fixed at intervals from 2 to 13 days after treatment.

In the two animals of the first group which did not display growth, it was seen after sectioning that in both a large flask-shaped ependymal sac had been formed. In one the blastema consists only of a dense mass of undifferentiated cells in which are large sinus-like blood-vessels. In the other a more normal blastema is present in which a myogenic zone of denser cells is recognizable. The ependymal sac is densely clothed with fibres of the white matter, some of which branch outwards in an irregular fashion through the blastema. Schwann cells are noticeably deficient, but pigment cells are abnormally abundant.

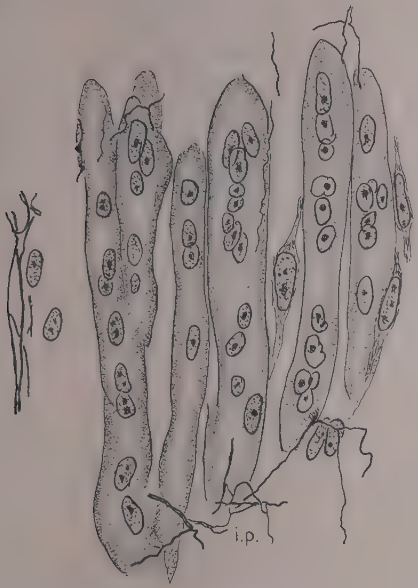
Of the group of nine regenerates which grew at sub-normal rates under the influence of the nitrogen mustard, five examples were fixed and sectioned at lengths which varied from 0·5 to 4·0 mm. In all of them the component tissues of the normal regenerates were present, though with some degree of distortion in texture and arrangement. The degree of histological development corresponds with that of normal examples of the same age irrespective of the sub-normal length of the treated regenerates.



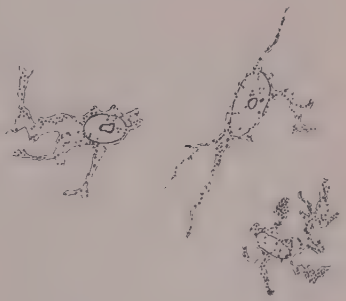
TEXT-FIG. 12. Part of cut surface of bisected 9·5-mm. tail regenerate of *Sphaerodactylus* showing the result of a diazonium reaction for esterases. The coloured reaction product is confined to the end regions of the muscle fibres. ×40.

One feature again common to this group of regenerates was a superabundance of melanocytes. At the tip they are present in such excess as to largely obscure everything else. In a 1·5-mm. regenerate (25 days) appearances suggest that the pigment cells are migrating along the ependymal tube towards the tip and out beyond it at the open end of the surrounding cartilage. The base of the tube is also surrounded by melanocytes, many of which do not yet contain pigment granules at their full density. Elsewhere in the regenerate they are more than usually abundant in the loose parenchyma between the cartilaginous tube and the muscle zone. Into both of these differentiated structures occasional melanocytes have penetrated. In younger members of this series (0·5 mm., 15–17 days), the blood-vessels are dilated into large sinuses. There are also numerous large empty lacunae within the mesenchyme of the regenerate. In both of these examples nuclear effects of the nitrogen mustard are apparent in the presence of both pycnotic nuclei and cells in abnormal mitosis with scattered chromosomes unattached to a mitotic spindle.

Within the dorsal root ganglia of the stump the effect of fixatives on the perikarya associated with the regeneration described above (p. 292) is seen to a greater extent than in normal regeneration. Some of the corresponding motor-cells within the cord are also affected. However, there was no evidence at any stage of the degeneration of neurones under the influence of the nitrogen mustard such as Hughes & Tschumi (1958) have demonstrated in the immature larval spinal ganglia of *Xenopus laevis*.



TEXT-FIG. 13. Parts of a terminal muscle segment of a 7-mm. regenerate of *Sphaerodactylus* 7 days after treatment with 1 : 1,000 nitrogen mustard. The intersegmental plexus of motor nerves (*i.p.*) is almost entirely without Schwann cells. × 546.



TEXT-FIG. 14. Young melanocytes within the parenchymatous connective tissue of a 5-mm. tail regenerate of *Sphaerodactylus* 5 days after treatment with 1 : 1,000 nitrogen mustard. Pigment granules are still separately discernible. × 1,233.

In the second series of experiments the substance was applied to regenerates 4-5 mm. long in which the constituent tissues are already largely differentiated. Two days after application the first effects of the treatment have already spread throughout the regenerate. Pycnotic nuclei are seen most commonly in the muscle bands, the ependymal tube, and the epidermis. The nerve fibres round the ependymal tube no longer reach to its tip, and counts in transverse sections of the total number of fibres in the longitudinal trunks indicate a considerable thinning out in number at the tip of the regenerate though some fibres are still to be seen within the epidermis. In all tissues the nitrogen mustard has affected either the cell surfaces or the intercellular matrix in such a way that contact between cells has been partly lost. The spaces between them are everywhere enlarged. On the 4th day after treatment the longitudinal nerve trunks share in this effect by assuming a characteristic frayed-out appearance. Between the fibres, Schwann cells are seen in degeneration (Plate, fig. F). In the succeeding days their rarity among the smaller bundles of nerve fibres is a striking feature (Plate, fig. H; Text-fig. 13). Elsewhere, from 4 days onwards, tissues begin to resume a more normal aspect although pycnotic nuclei remain common. The nerve fibres at the tip of the regenerate are soon restored. Nuclei of the outer layers of the *stratum corneum* become shrivelled and gradually sealed off from the healthy cells within by thick laminae of keratin. The dead material is sloughed off by the 6th day and the whole epidermis thereby becomes much thinner. Mitotic figures reappear among the cells of the *stratum germinativum* (Plate, fig. H), the nuclei of which are abnormally large though their mitotic figures do not suggest a polyploid condition.

On the 5th day the influence of the nitrogen mustard on the production of melanin becomes apparent. The new keratinous zone of the epidermis is uniformly black. Within the dermis, melanocytes are no less conspicuous than usual, but some of them are extending long-cell processes into the mesenchymatous tissue beneath, within which are also to be seen cells with nuclei similar to those of the surrounding fibroblasts but from which there radiate fine cytoplasmic filaments in which small pigment granules are sparsely distributed (Text-fig. 14). These are apparently cells which are differentiating into melanocytes. On the 7th day the cytoplasm of most cells which contain pigment is a uniform dense black (Plate, fig. H) though occasionally fibroblast-like elements containing a few granules are to be seen. The general proportion of melanocytes increases still further in succeeding days: in one specimen there is much extracellular pigment within the mesenchyme 13 days after treatment; young melanocytes are, however, still to be seen.

This apparent influence of nitrogen mustard in evoking the formation of melanin within mesodermal cells does not seem to be confined to the regenerate which was originally treated with the substance. In one animal a regenerate of 1·5 mm. grew in 25 days, 20 days after treatment. This was removed, together with two caudal segments of the original tail, and a further regenerate allowed to develop without further treatment. It grew to 3·5 mm. in 21 days. On sectioning it was found that melanocytes were abnormally abundant both around the ependymal tube and within the general parenchyma.

The effect of nitrogen mustard at the concentrations here used is apparently confined to young and rapidly growing regenerates. Two older examples were treated, one 10-mm. long, and the other so mature that the boundary between new and old tail was no longer traceable. On sectioning, no effect of the treatment was seen in either instance.

DISCUSSION

Hitherto, the regeneration of the lizard's tail has been discussed largely in terms of the anatomy of the regenerate and the latter's relationship with the normal tail. We can now turn to the further question of the development and histogenesis of the new tail in comparison with the corresponding processes in normal embryology.

From the regenerating blastema arise cells that form cartilage, muscle fibres, general mesenchyme, as well as Schwann cells and melanocytes. In ontogeny the neural crest is the main source of these latter two cell types, though there is evidence that in some vertebrates Schwann cells are not exclusively derived from the neural crest (Hörstadius, 1950, p. 31). As far as it is known the neurones of the dorsal root ganglia develop wholly from neural crest cells; yet in the regeneration of the tail of a Urodele fresh ganglia are formed by the outward migration of cells from the new spinal cord (Duesberg, 1925).

The regenerative blastema arises in the first place in close association with the ependymal sac. It is possible that cells which originate by mitosis within this neural epithelium may be in genetical continuity with those which at an early embryonic stage were formed together with the neural crest. A possible difficulty here is that, in a bifid tail, one branch may lack a cartilaginous tube and its contained ependymal prolongation. Brindley (1898) has described such an instance in *Mabuia carinata*, and Woodland (1920) has evoked this condition experimentally in *Hemidactylus flaviviridis*. However, nothing is yet known in such examples about the distribution or the origin of pigment cells or even how the growth of the accessory branch proceeds in the first instance.

In the regenerating tail of *Sphaerodactylus* the first melanocytes originate from the pigmented meningeal investment of the spinal cord, but the experiments with nitrogen mustard described in this paper show that some normally unpigmented cells within the general mesenchyme have the potentiality of forming melanin. This observation is paralleled by the experiments of Figge (1948), who found that in tadpoles of *Rana* which had been treated with indophenol dyes, melanin granules appeared within connective tissue-cells of the larva.

Woodland (1920), in discussing the differences between the normal and regenerated tails of a lizard, regards the latter as a 'cheap "jerry-built" structure only reproduced for the purpose of being shed'. The analogy has some value in relation to the respective time-scales on which each structure is laid down. Normal development in the Lacertilia is extremely slow, and extends over several months, whereas tail regeneration is complete within a few weeks of adult life. It is in this light that the different modes of histogenesis of normal and regenerated muscle are best viewed, irrespective of the relative worth of the final products, in which no difference is apparent on microscopical examination.

In the regenerate, the division into myotome-like segments persists during and after the formation of muscle fibres. Motor nerve fibres enter the developing musculature at intersegmental planes, to which a positive esterase reaction at neuromuscular junctions is at first restricted. The normal development of muscle fibres in *Sphaerodactylus* is, however, similar to that of other Amniotes in that the original segmentation breaks down before the differentiation of muscle fibres, and when motor fibres enter the musculature the myocommata have long been unrecognizable.

Why one type of myogenesis should necessarily be more rapid than the other is yet impossible to explain, but the thesis that each is correlated with a different rate of ontogeny receives support from the corresponding developments in the tadpole of the Anuran *Xenopus*, where the crucial events are compressed into a few hours. Here, as Lewis & Hughes (1957) and Lewis (1958) have shown, the histogenesis of the trunk muscles is closely comparable to that in the Lacertilian tail regenerate; segmental divisions are retained, innervation is at first confined to the ends of the muscle fibres, and esterases first appear in the myocommatal planes. Further exploration of this subject demands an extended comparative survey of the functional aspects of myogenesis.

SUMMARY

1. Tail regeneration in the Geckonid Lizards *Sphaerodactylus goniorhynchus* and *argus* is described. The rate of growth of a first regenerate, after a latent period of 10–12 days, is about 0·47 mm. per day. Subsequent regenerates grow more slowly.

2. The tissues of the new tail are derived from a regenerative blastema which is formed beneath the epidermis covering the broken surface of the tail. A flask-shaped outgrowth of the ependymal epithelium is a centre for the proliferation of new cells.

3. The outer part of the regenerative blastema is a zone of myogenesis, which becomes divided into the same number of muscle bands as in the original tail. Each muscle band becomes segmented, and motor nerves enter the intersegmental planes. At these sites a positive reaction for esterases is first seen. Multinucleate muscle fibres, which stretch from one intersegmental plane to the next, arise by the fusion of myoblastic cells.

4. The regenerate is innervated by branches of the last three remaining pairs of spinal nerves. These branches keep pace with the growth of the regenerate, and form longitudinal nerves. Their terminal fibres enter the new ectoderm on the 4th day.

5. Nitrogen mustard applied in solution to the outer surface of the regenerating area may inhibit, or slow down, the new growth. Degeneration of each type of cell within the regenerate is caused by nitrogen mustard, but melanocytes become more abundant than normal.

6. A regenerate subsequent to one inhibited by nitrogen mustard grows as

fast, or even faster, than a normal first regenerate, and again contains an excess of melanocytes.

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EXPLANATION OF PLATE

Fig. A. Spinal nerve roots of tail of *Sphaerodactylus* which send fibres into a 6-day regenerate. Ventral root fibres run vertically in figure, dorsal root fibres emerge horizontally from ganglion on right. Ventral root fibres are more swollen than those of a dorsal root. Nuclei of perikarya of ganglion much vacuolated at fixation. Bodian. × 340.

Fig. B. Orientated mitotic figures among myoblasts in 2·5 mm. regenerate (17 days) of *Sphaerodactylus*. H. & E. × 340.

Fig. C. End stages of pycnosis round muscle fibres in 6-mm. regenerate of *Sphaerodactylus*. H. & E. × 340.

Fig. D. Fibres from longitudinal nerves among cells of inner layers of epidermis in 4-day regenerate of *Sphaerodactylus*. Holmes. × 340.

Fig. E. Motor fibres in intersegmental plane (horizontally across middle of figure) at base of muscle band in 1·5-mm. regenerate (15 days) of *Sphaerodactylus*. Holmes. (Same specimen as in Text-fig. 6.) × 340.

Fig. F. Longitudinal nerve in 3-mm. regenerate of *Sphaerodactylus* 4 days after treatment with 1 : 1,000 nitrogen mustard, showing degeneration of Schwann cells and fraying of nerve fibres. Bodian. × 340.

Fig. G. Part of dorsal root ganglion supplying 1-mm. regenerate (11 days) of *Sphaerodactylus* showing infiltration with lymphocytes. Nuclei of perikarya fixed without vacuolation. H. & E. × 340.

Fig. H. Area of dermis and epidermis (top left) from 7-mm. regenerate of *Sphaerodactylus*, 7 days after treatment with 1 : 1,000 nitrogen mustard, showing peripheral nerves with very few Schwann cells, frayed nerve trunk (bottom right), and new melanoblasts. Among the large epidermal nuclei is one in metaphase. To be compared with Fig. I. Bodian. × 340.

Fig. I. Area of dermis and epidermis (top left) of 7-mm. regenerate of *Sphaerodactylus* to serve as control to Fig. H. Note smaller size of epidermal nuclei and numerous Schwann cells on peripheral nerve branches. Bodian. × 340.

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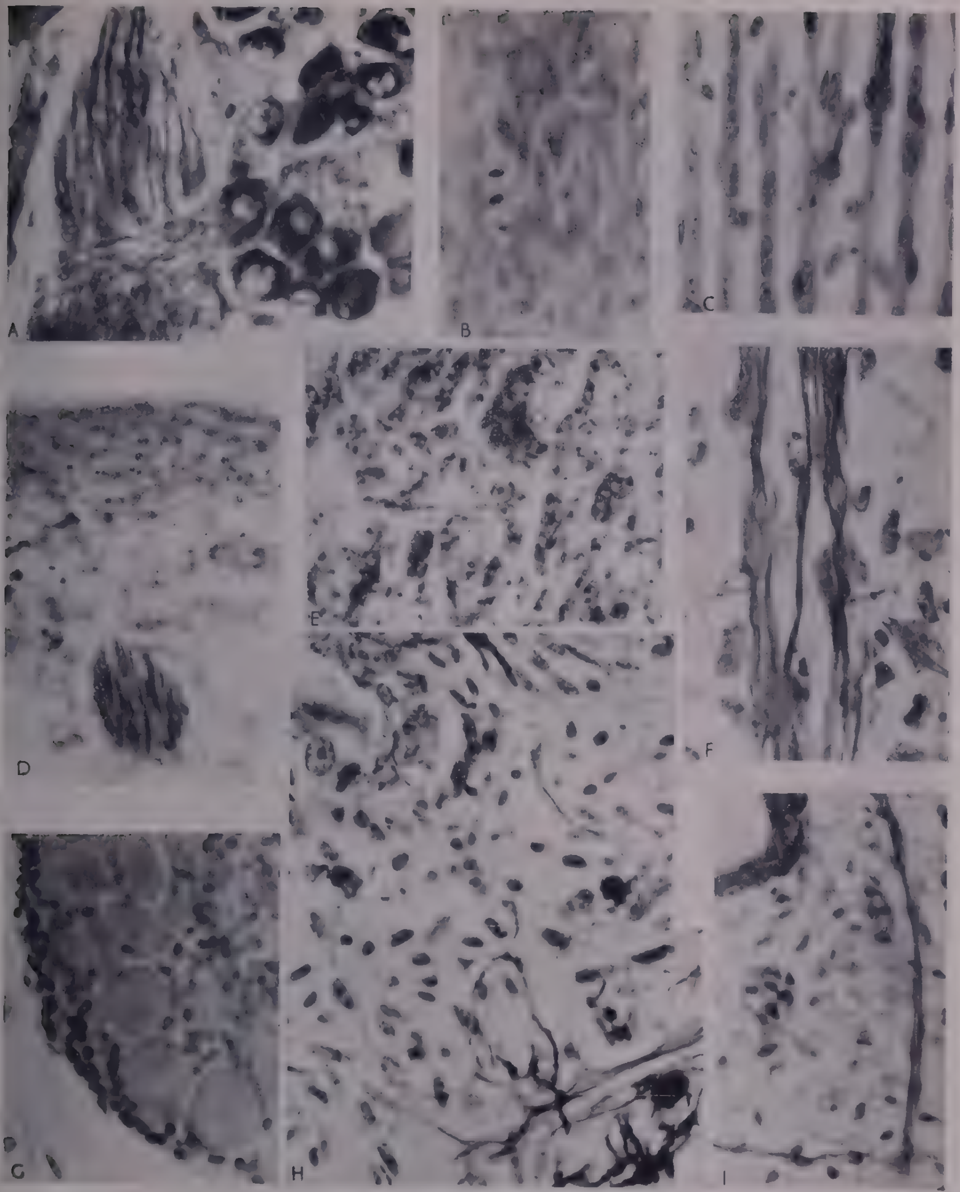

A. HUGHES *and* D. NEW

The Development of the Cornea in the Chick

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WITH TWO PLATES

INTRODUCTION

THE present study was undertaken in order to ascertain the miscroscopical appearance of the cornea of the chick at each developmental stage of the whole embryo. The early development of the cornea, namely that up to stage 25, has been included in an account of the eye as a whole (O'Rahilly & Meyer, 1959) and the present paper is a continuation of the study up to the time of hatching. For purposes of comparison, the cornea of the adult has also been examined.

Existing accounts of the embryonic development of the eye have been rendered inadequate by recent refinements in the staging of mammalian and avian embryos. The system of stages devised by Hamburger & Hamilton (1951) has been employed in the present investigation. This classification, which is not based on chronological age, was made to depend on external morphological appearances only.

MATERIAL AND METHODS

The chick embryos utilized in the present investigation were treated in the manner described previously (O'Rahilly & Meyer, 1959). In the case of some of the older embryos, however, decalcification in a solution of formic acid and sodium citrate was employed prior to embedding. Serial sections of the heads or of the isolated eyes of 108 embryos between stage 26 and hatching, and of the corneae of two adults, were investigated. The number of specimens examined at each stage is given in parentheses in the following list:

> Stage 26 (8 specimens), 27 (5), 28 (16), 29 (5), 30 (13), 31 (7), 32 (7), 33 (6), 34 (15), 35 (4), 36 (6), 37 (6), 38 (2), 39 (1), 40 (4), 42 (1), newly hatched (2), adult (2).

The measurements given throughout this paper are based on stained sections and are, therefore, subject to the alterations in size that result from fixation and subsequent histological procedures.

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SUMMARY OF EARLY STAGES

The early development of the cornea (up to stage 25) may be summarized as follows.

At stage 15, when a lens pore is still present, a mass of fibrils (anhistic mass of Kessler, anterior vitreous body of v. Lenhossék, mesostroma of Studnička) was observed within a small triangular area, the anlage of the aqueous chamber, bounded by the surface ectoderm, the lens, and the lip of the optic cup. At the next stage a fine membrane (directional membrane of Knape, second membranous corneal veil of Laguesse, principal ribbon of Redslob), which stained selectively with aniline blue, appeared in this space, deep and parallel to the surface ectoderm and connected with the mesenchyme around the optic cup.

Closure of the lens pore and detachment of the lens occurred at stage 17. The surface ectoderm now formed a continuous anterior epithelium for the future cornea and rested on a basement membrane. The directional membrane was still evident deep to the epithelium.

Approximately a day later, at stages 21–22, mesenchymal cells from the margin of the optic cup commenced to advance centrally along the proximal or deep surface of the directional membrane. These cells were the beginning of the mesothelial lining of the cornea. The directional membrane was still identified by stage 24, but its later fate was difficult to determine with certainty. By stage 25, when the mesothelium formed a continuous lining for the cornea, an acellular, seemingly fibrillar layer (*cornea propria* of Kessler, postepithelial layer of Hagedoorn, spongioid layer of Ladijenski) appeared between the mesothelium and the anterior epithelium. This layer, which contributed later to the formation of the *substantia propria*, was stained selectively with aniline blue or with fast green.

Thus, by stage 25, the cornea of the chick comprised an anterior epithelium, an acellular postepithelial layer, and a mesothelium.

PRESENT OBSERVATIONS

Stage 26 (4½–5 days). (Plate 1, fig. A.) The structure of the cornea at this stage closely resembled that seen at stage 25. The anterior epithelium possessed a distinct basement membrane that displayed an affinity for fast green. The postepithelial layer (Plate 1, fig. A, arrow) in most embryos at this stage was equal in thickness to the overlying surface ectoderm, except peripherally, where it gradually tapered to terminate a short distance peripheral to the rim of the optic cup. As in the previous stage, the postepithelial layer appeared as an acellular, fibrillar, or lamellar band. It was stained selectively by fast green or by aniline blue and the fibrils or lamellae often presented a wavy appearance. In one specimen the postepithelial layer was clearly bounded posteriorly by a membrane that may have been either the directional membrane characteristic of earlier stages or a newly formed basement membrane for the mesothelium. In sections, the mesothelium appeared as a single row of spindle-shaped cells (their long axes being parallel with the surface) that constituted a simple squamous epithelium. The mesothelium formed a complete lining for the cornea.

Stage 27 (5 days). (Plate 1, fig. B.) The postepithelial layer of the cornea of most embryos at this stage had become thicker and a few mesenchymal cells, the corneal 'corpuscles', had now begun to invade it from the periphery, the extent of the penetration varying from only a few cells at the periphery to an almost continuous layer of cells occupying the entire posterior part of the postepithelial layer.

Stage 28 ($5\frac{1}{2}$ days). The cornea now measured between 25 and 50 μ in thickness. As in the previous stage, the density and extent of migration of the corneal corpuscles varied widely. In some specimens the postepithelial layer was infiltrated with the mesenchymal cells; in others they were absent. When present, the corpuscles appeared to occupy the entire thickness of the postepithelial layer except for a narrow acellular band anteriorly. The posterior portion may now be termed the *substantia propria* of the cornea, whereas the anterior band is the uninvaded remains of the postepithelial layer. The anterior band was bounded in front by the basement membrane of the anterior epithelium. The anterior epithelium possessed a distinct basement membrane that seemed to be composed of compact fibrils of the postepithelial layer. This was particularly evident in specimens in which the anterior epithelium had been artificially detached from the postepithelial layer. In such cases, the basement membrane remained in contact with the anterior band and actually appeared to be a part of it.

The cells of the mesothelium had become situated somewhat closer together and, in most embryos at this stage, were separated from the *substantia propria* by a distinct basement membrane. In sections stained by the Papanicolaou technique, this membrane appeared to be formed of a group of compact green fibrils. Remnants of the directional membrane were detected in two specimens at this stage.

Stage 29 (6 days). (Plate 1, fig. C.) The postepithelial layer was heavily infiltrated with corneal corpuscles and in only one specimen did they fail to reach the centre of the cornea. The acellular anterior band of the postepithelial layer (Plate 1, fig. C, arrow) was easily recognizable and displayed an affinity for fast green. Anteriorly, it was closely associated with the basement membrane for the anterior epithelium, whereas peripherally it extended a considerable distance beyond the limit of the mesothelium of the anterior chamber. The mesothelium, low cuboidal in type, was bounded anteriorly by a basement membrane.

Stage 30 ($6\frac{1}{2}$ days). (Plate 1, fig. D.) The appearances resembled those seen in the preceding stage. The postepithelial layer, however, was more heavily infiltrated with corneal corpuscles and in only one specimen out of 13 examined did the cells fail to reach the centre of the cornea. The anterior band of the postepithelial layer was still evident and showed an affinity for fast green. Its thickness was about equal to the height of the anterior epithelium. Distinct basement membranes for both the anterior epithelium and the mesothelium were visible. The mesothelium was still low cuboidal in type.

Stage 31 (7 days). The cornea resembled that seen in the previous stage. The anterior acellular band of the postepithelial layer had decreased in thickness and now measured about one-half of the height of the anterior epithelium. It appeared to be composed of closely packed fibrils that possessed an affinity for fast green. The *substantia propria* had consequently increased in size and had assumed a more fibrillar or lamellar character. The corneal corpuscles were distributed uniformly throughout the *substantia propria.* In the specimens stained with Papanicolaou's technique, a narrow, green, acellular, fibrillar band was observed immediately in front of the mesothelium. Peripherally this posterior band formed the boundary of the so-called 'stratified endothelium'. Its appearance and staining characteristics resembled those of the anterior band of the postepithelial layer. The basement membrane of the mesothelium appeared to be a part of this fibrillar band.

Stage 32 (7½ days). (Plate 1, fig. E.) The cornea now measured about 100 μ in thickness and had undergone very little change. The anterior band of the postepithelial layer retained its same relative thickness and consisted of wavy fibrils that showed an affinity for aniline blue or for fast green. The mesothelium was simple cuboidal in type and was bounded anteriorly by a thin basement membrane that possessed an affinity for aniline blue or for fast green.

Stage 33 (7½–8 days). The appearances seen at this stage resembled very closely those seen at stage 32. The anterior band of the postepithelial layer, however, had become somewhat thinner.

Stage 34 (8 days). (Plate 1, fig. F.) The anterior band of the postepithelial layer varied from one-eighth to one-half of the thickness of the anterior epithelium. The fibrils that composed it had become more refringent and condensed, and were stained brilliantly with aniline blue or fast green. In some specimens a similar band of brilliantly stained refringent fibrils or lamellae was noted condensed against or incorporated with the basement membrane of the mesothelium.

The fibrils or lamellae of the *substantia propria* appeared to possess a greater affinity for aniline blue or fast green than they had previously. By the use of Bodian's technique, nerve-fibres were demonstrated in the peripheral region of the *substantia propria* near the 'stratified endothelium'. The tissue immediately behind the last-mentioned appeared very loose ('cellules clairsemées', Gabriélidès, 1895). This loose area was presumably the beginning of the interciliary sinus or cilioscleral space (Wolff, 1954).

Stage 35 (8–9 days). The cornea measured about 175 μ in thickness. The anterior band of the postepithelial layer was very thin, amounting to only about one-eighth of the height of the anterior epithelium. The fibrils or lamellae of the *substantia propria* were wavy, more intensely stained, and appeared to be intertwined with those of the anterior band. The posterior band was no longer evident. The mesothelium was bounded anteriorly by only a thin basement membrane, which, like the anterior band of the postepithelial layer, appeared to send out fibrils or lamellae into the *substantia propria*. The mesothelium had undergone very little change and was still composed of a single layer of cuboidal cells.

Stage 36 (10 days). (Plate 2, fig. G.) The anterior band of the postepithelial layer was no longer evident and apparently had become invaded by the corneal corpuscles. Consequently, the *substantia propria* comprised all the tissue between the basement membrane of the anterior epithelium and that of the mesothelium. The basement membrane and the fibrils or lamellae of the *substantia propria* were selectively stained with aniline blue or with fast green. Nerve-fibres were observed with Bodian's method in the middle of the peripheral portion of the *substantia propria*.

Stage 37 (11 days). The appearances resembled those seen at the previous stage. A prominent nerve-bundle was detected in the peripheral region of the *substantia propria* (sclerocorneal junction) by Bodian's technique. It divided into numerous branches that were distributed to the iris, the 'stratified endothelium', and the *substantia propria*. The nerve-fibres in the *substantia propria* were located in its middle portion and did not appear to extend as far as the centre of the cornea.

Stage 38 (12 days). The anterior epithelium, which, since stage 17, generally contained 3 nuclear layers, now presented 4; the most anterior layer was still squamous. The anterior portion of the *substantia propria* stained more intensely with aniline blue than the posterior portion.

Stage 39 (13 days). The anterior epithelium presented 4–5 nuclear layers, of which the most superficial was squamous. The anterior portion of the *substantia propria* stained more intensely with fast green but this denser part now occupied only about one-eighth of the entire thickness of the cornea. A second dense layer was discerned faintly at about the middle of the *substantia propria*. Beneath the basement membrane of the anterior epithelium, at least in some areas, a narrow, acellular, green band was observed. This is believed to be the beginning of the anterior limiting lamina (Bowman's membrane) of the cornea.

Stage 40 (14 days). (Plate 2, figs. H, I.) The cornea now measured about 250 μ in thickness. The second dense layer in the *substantia propria* was better defined. The anterior limiting lamina showed as a narrow but definite layer immediately beneath the basement membrane of the anterior epithelium (Plate 2, fig. H). It stained intensely with aniline blue. The mesothelium was still simple cuboidal and was sharply delimited from the *substantia propria* by a distinct basement membrane (Plate 2, fig. I).

Stage 42 (16 days). The cornea measured about 300 μ in thickness. The anterior limiting lamina stained intensely with fast green.

Newly hatched. (Plate 2, figs. J, K.) The cornea ranged from about 250 to

350 μ in thickness. The anterior epithelium (Plate 2, fig. K), about 30 to 40 μ in thickness, was stratified squamous in type and comprised two zones. The superficial zone contained 3–4 layers of flat nuclei and its cytoplasm appeared purplish with the Mallory–Masson technique. The deep zone contained about 3 layers of round or oval nuclei and appeared reddish, particularly in its most posterior portion. The anterior epithelium was limited by an argyrophilic basement membrane, as seen with Bodian's technique.

The anterior limiting lamina (Plate 2, fig. K) was a narrow refractile band that stained selectively with aniline blue or with fast green. It appeared to be only about 2 μ in thickness. It was not sharply demarcated from the subjacent *substantia propria*, the anterior one-sixth of which also stained deeply. A second dense layer in the *substantia propria*, as seen at some of the earlier stages, was indefinite. The lamellae of the *substantia propria* stained with aniline blue or with fast green.

The mesothelium of the anterior chamber (Plate 2, fig. J) was simple cuboidal in type and rested on an argyrophilic basement membrane.

Adult (Plate 2, figs. L, M.) The cornea was approximately 450 μ in thickness. The anterior epithelium, about 30 μ in thickness, comprised two zones (Plate 2, fig. M). The superficial zone contained about 3 layers of flat nuclei and appeared a brilliant red with the Mallory–Masson technique but a yellowish orange with Papanicolaou's method. The deep zone contained about 4 layers of round or oval nuclei that presented eccentric, reddish areas with Masson's stain, whereas the cytoplasm appeared bluish. With Papanicolaou's method, however, the cytoplasm of the more anterior cells appeared greenish, and that of the posterior columnar cells was brownish. The cytoplasm adjacent to the basement membrane appeared reddish in places with Mallory–Masson's method, and yellowish-orange with that of Papanicolaou's.

The anterior limiting lamina was a thin band that stained with aniline blue or with fast green. It measured perhaps some 5 μ or more in thickness, but it was not separated sharply from the underlying *substantia propria*. The *substantia propria* lacked any definite denser bands and it contained relatively far fewer nuclei than did the cornea in the newly hatched chick.

The mesothelium of the anterior chamber was either cuboidal or squamous in type. A narrow homogeneous band, which stained with aniline blue, was observed beneath the mesothelium (Plate 2, fig. L, arrow). This was believed to be the posterior limiting lamina (Descemet's membrane) of the cornea. It measured about 4 μ in thickness. Considerable difficulty was experienced in deciding, by means of the Mallory–Masson technique, whether a basement membrane was present in addition to the limiting lamina. Some slight suggestions of a delineation between the posterior limiting lamina and the *substantia propria* were observed at intervals.

In brief, the cornea of the adult *Gallus domesticus*, like that of man, comprises the following layers: (1) an anterior epithelium, resting on a basement membrane, (2) an anterior limiting lamina, (3) a *substantia propria*, (4) a posterior limiting lamina, and (5) a mesothelium.

The present authors' observations on the development of the cornea are summarized in Table 1.

TABLE 1

Summary of the development of the cornea in the chick

Stage	*Feature*
15	Anhistic mass.
16	Directional membrane.
17	Detachment of lens from surface ectoderm.
	Continuous anterior epithelium for future cornea.
21–22	Mesenchymal cells (mesothelium) grow along directional membrane.
25	Postepithelial layer between anterior epithelium and mesothelium.
	Continuous mesothelial lining for cornea.
26–29	Basement membrane for mesothelium can be detected.
27	Mesenchymal cells (corneal 'corpuscles') begin to invade postepithelial layer.
28	Postepithelial layer subdivided into anterior band and *substantia propria*.
29	Corneal 'corpuscles' reach centre of cornea.
31	Posterior band appears.
35	Posterior band no longer evident.
36	Anterior band no longer evident.
39	Anterior limiting lamina appears.
After hatching	Posterior limiting lamina develops.

DISCUSSION

The sequence of histogenesis of the chick cornea has been well established primarily from the detailed descriptions of Laguesse (1926), Redslob (1935), and Watzka (1935); in general, the observations of these authors have been corroborated in the present study. However, the fact that these and other investigations of corneal development have been based on chronological age renders the data inadequate for the accurate pinpointing of the sequence of histogenetic and morphogenetic alterations.

The layers of the cornea will now be discussed *seriatim*.

Anterior epithelium. The anterior epithelium of the cornea is derived from the surface ectoderm and forms a continuous epithelial layer immediately after detachment of the lens vesicle (stage 17). Initially it consists of 3 nuclear layers bounded internally by a distinct basement membrane. The anterior epithelium presents 4 nuclear layers at stage 38, and 4–5 at stage 39. The stratification of the epithelium at the time of hatching is similar to that observed in the adult, namely about 7 nuclear layers bounded internally by a distinct basement membrane.

Kessler (1877) observed only two nuclear layers, a superficial squamous and a deep cylindrical, both of which persisted almost to the end of embryonic development, at which time a third layer of round cells appeared. Owing to the relative slowness of its development, Kessler considered the anterior epithelium as the last portion of the cornea to complete its development. Redslob (1935) reported two layers of epithelial cells at 135 hours, 3 at 15 days, 4 at 18 days, and 5 cellular layers several days after hatching.

It is not generally realized that the anterior epithelium possesses its own basement membrane (Busacca, 1949) distinct from the anterior limiting lamina, although these 3 layers were clearly figured by Bach & Seefelder (1914, Tafel 27, fig. 3). The basement membrane is derived from that of the surface ectoderm and, in a certain sense, is therefore present at all stages in the development of the eye.

Anterior limiting lamina. In the present study the anterior limiting lamina (Bowman's membrane) is first recognizable at stage 39 as a new, acellular band situated immediately subjacent to the basement membrane of the anterior epithelium. Merely from its location and appearance, it could not be distinguished from the defunct anterior and acellular remains of the postepithelial layer. These findings are in close agreement with those of Redslob (1935), who detected the complete disappearance of the anterior zone of the postepithelial layer at 9 days and the appearance of the anterior limiting lamina at 9½ days.

The majority of investigators (Kessler, 1877; Angelucci, 1881; Knape, 1909, 1910; Laguesse, 1923*a*, 1926; Hagedoorn, 1930; Jasswoin, 1939; Mann, 1931, 1949), however, have failed to note the extremely transient disappearance of the anterior band and consequently have contended that the anterior acellular zone of the postepithelial layer persisted into the adult as the anterior limiting lamina. The fact that these investigators did not examine a sufficiently closely graded series of embryos probably accounts for this misconception. It is of interest, in this regard, to cite the observations of Collins (1897) that 'in mammals [human and mice] there is a stage in the development of the cornea where no elastic membrane is present'.

Unlike the basement membrane of the anterior epithelium, the anterior limiting lamina is absent in many species of animals (Calmettes *et al.*, 1956).

Substantia propria. It has been confirmed by most authors that the *substantia propria* of the cornea is derived from a primitive acellular layer (*cornea propria* of Kessler, 1877, and Angelucci, 1881; anterior vitreous body of Knape, 1909, 1910, and Watzka, 1935; mesostroma of Laguesse, 1926, and Mann, 1931; postepithelial layer of Hagedoorn, 1930) and from mesodermal cells (corneal 'corpuscles') that invade it secondarily from the peripheral mesenchyme. By stage 26 the postepithelial layer is evident as an acellular fibrillar (Neuschüler, 1931; Redslob, 1935; Watzka, 1935; Jasswoin, 1939) or lamellar (Ladijenski, 1915; Laguesse, 1923*a*) band between the anterior epithelium and the mesothelium. The infiltration of the postepithelial layer by corneal corpuscles is first noted at stage 27 and, by stages 29–31, the invading mesodermal cells have formed a continuous cellular layer, the *substantia propria*, which occupies the entire extent of the postepithelial layer except for an anterior, narrow, acellular band subjacent to the anterior epithelium.

Initial mesenchymal invasion was detected by several authors at 5 or 6 days of incubation (125 hours: Redslob, 1935; 144 hours: Knape, 1909, 1910; towards the 7th day of incubation: Jasswoin, 1939). A continuous cellular layer was first observed by Kessler (1877) on the 8th day, and by Laguesse (1926), Watzka (1935), and Redslob (1935) by the 6th day, at which time the cells supposedly assumed their definitive form (Levi, 1926).

With further development the *substantia propria* increases in thickness and gradually assumes a more fibrillar or lamellar character as revealed by aniline blue or fast green. Laguesse (1923*b*) and Watzka (1935) observed definitive fibrils (precollagen, according to the former) in the *substantia propria* during the 7th day of incubation, which corresponds, in time, to fibril formation in other mesenchymal tissues of the chick embryo (Dantschakoff, 1908). Ladijenski (1915) reported the presence of collagen during this time. The definitive pattern of stromal collagenous fibres begins to form on about the 8th day and is well established by the 14th (Coulombre & Coulombre, 1958 *b*, *d*). Jasswoin (1939) contended that the fibrils pass through an argyophilic phase before becoming collagenous at 10–12 days.

Concomitant with the increase in thickness of the *substantia propria*, the anterior, acellular remains of the postepithelial layer become more fibrillar or lamellar (stage 34) and progressively thinner and more compact, apparently owing to the gradual encroachment of the corneal corpuscles, and they completely disappear by stage 36. As development proceeds, more intensely stained bands of fibrils or lamellae become evident in the *substantia propria* (stages 39–40). According to Coulombre & Coulombre (1958 *a*, *c*, *d*), metachromatic mucopolysaccharide is first detectable in the corneal stroma on the 14th day, at which time the cornea begins to become transparent, to accelerate its rate of dehydration (begun on about the 9th day), and to become thinner. By the 18th or 19th day these authors (1958 *a*, *c*) noted that the metachromasia had spread to all parts of the stroma and that the cornea had assumed its adult transparency. Studies of developing human, rat, mouse, guinea-pig, and rabbit corneae (Aurell & Holmgren, 1953) revealed the development of metachromasia 'just at the time when the eye had to start fulfilling its optical functions'.

Posterior limiting lamina. The posterior limiting lamina (Descemet's membrane) has not been identified with certainty in any of the embryonic corneae examined in the present series. It is evident in the adult cornea, however, as a narrow, homogeneous band, about 4 μ in thickness, and it possesses an affinity for aniline blue.

Many different opinions exist regarding the origin of the posterior limiting lamina. The data provided in the present study support the findings of Redslob (1935), who failed to detect this lamina during embryonic development. He did not identify the posterior limiting lamina until 12 days after hatching, at which time it appeared as a very narrow, homogeneous, refringent zone, separating the mesothelium from its basement membrane. Initially it was discontinuous, completely absent centrally, but with subsequent growth it formed a continuous lamina (18 days) and attained its definitive form at 90 days after hatching. Redslob pointed out that the supposed identification of this lamina during embryonic life could be attributed to the fact that it is often confused with the basement membrane of the mesothelium. Electron microscopic studies of the developing chick cornea, however, have failed to detect a basement membrane distinct from the posterior limiting lamina (Jakus, 1956). According to the latter author, 'in chick embryos, Descemet's membrane has not been identified with certainty until about the tenth day of incubation. It appeared first as small, isolated pads between the stroma and the endothelial cells. The pads then seemed to grow laterally until they met and finally fused.'

Watzka (1935) considered the posterior limiting lamina as the fibrillar basement for the mesothelium, and noted its first appearance at 13 days of incubation. In support of Angelucci (1881) and Hagedoorn (1930), he believed that this lamina was a derivative of the mesothelium. Knape (1909, 1910), on the other hand, maintained that it was formed at 7 days of incubation by the self-differentiation of the directional membrane.

Some observers (Kessler, 1877; Laguesse, 1923*a*, 1926; Jasswoin, 1939; Mann, 1931, 1949) have contended that the posterior limiting lamina originated in the same manner and at the same time as the anterior limiting lamina. They have reported the existence of a posterior, acellular band of the postepithelial layer immediately after the formation of the *substantia propria* and they maintained that, like the anterior zone, it remained uninvaded by corneal corpuscles and eventually formed the posterior limiting lamina. That the anterior and posterior limiting laminae are essentially different types of structures, however, has been confirmed by electron microscopy (Rouiller *et al.*, 1954; Jakus, 1956).

Mesothelium. The mesothelium forms a complete lining for the cornea from stage 25 onwards. In the newly hatched chick, the mesothelium is simple cuboidal in type and rests on an argyrophilic basement membrane. The basement membrane for the mesothelium becomes evident by stage 29, although it may be present a few stages earlier. It stains selectively with aniline blue or with fast green by stage 36.

Redslob detected a continuous basement membrane at 125 hours, Laguesse (1926) at 154 hours. According to the former it consisted, by 135 hours, of fine fibrils that were continuous with the fibrillar network of the *substantia propria.* Redslob noted that, at 15 days, the basement membrane was distinct only in places and was virtually absent in the centre of the cornea. It was not evident on the following day, apparently owing to the separation of its fibrils by plates of hyaline material advancing from the periphery and resting directly against the mesothelium. The basement membrane reappeared again a few days after hatching, became detached from the mesothelium at 12 days, and subsequently became separated from the mesothelium by a narrow, homogeneous zone, the anlage of the posterior limiting lamina. Thus Redslob contended that, beginning with the formation of the posterior limiting lamina, the latter always separated the basement membrane from the mesothelium. He observed this relationship in the adult. Perhaps the 'variably faint line' described by Wislocki (1952) in the eye of the monkey, between the posterior limiting lamina and the *substantia propria*, is a similar structure, although he also noted 'a delicate membrane' between the posterior lamina and the mesothelium.

SUMMARY

1. The development of the cornea in the chick, based on the microscopical examination of serial sections of 108 morphologically staged chick embryos between stage 26 and hatching, is described. A description of the adult cornea is also provided.

2. Correlations between the internal histogenesis of the cornea and the external morphology of the chick embryo have been established from the use of staged embryos, so that the probable state of development of the cornea can be assessed from surface appearances only.

3. The cornea of the chick, at stage 26, consists of an anterior epithelium, an acellular postepithelial layer, and a mesothelium. Mesenchymal cells (corneal 'corpuscles') begin to infiltrate the postepithelial layer at stage 27. By stages 29-31 these cells occupy a continuous layer, the *substantia propria*, which comprises the entire extent of the postepithelial layer except for an anterior, acellular band subjacent to the anterior epithelium. The anterior band becomes progressively thinner and disappears at stage 36, the *substantia propria* then extending across the entire distance between the anterior epithelium and the mesothelium. The anterior limiting lamina first appears at stage 39. It closely resembles and is often confused with the temporary anterior band of the postepithelial layer. The posterior limiting lamina was not identified with certainty in any of the embryonic corneae examined.

4. It has been stressed that embryological studies in which external staging and internal development are correlated provide a more precise interpretation of developmental processes, and are an essential prelude to experimental, physiological, biochemical, and histochemical investigations of embryonic development.

ACKNOWLEDGEMENT

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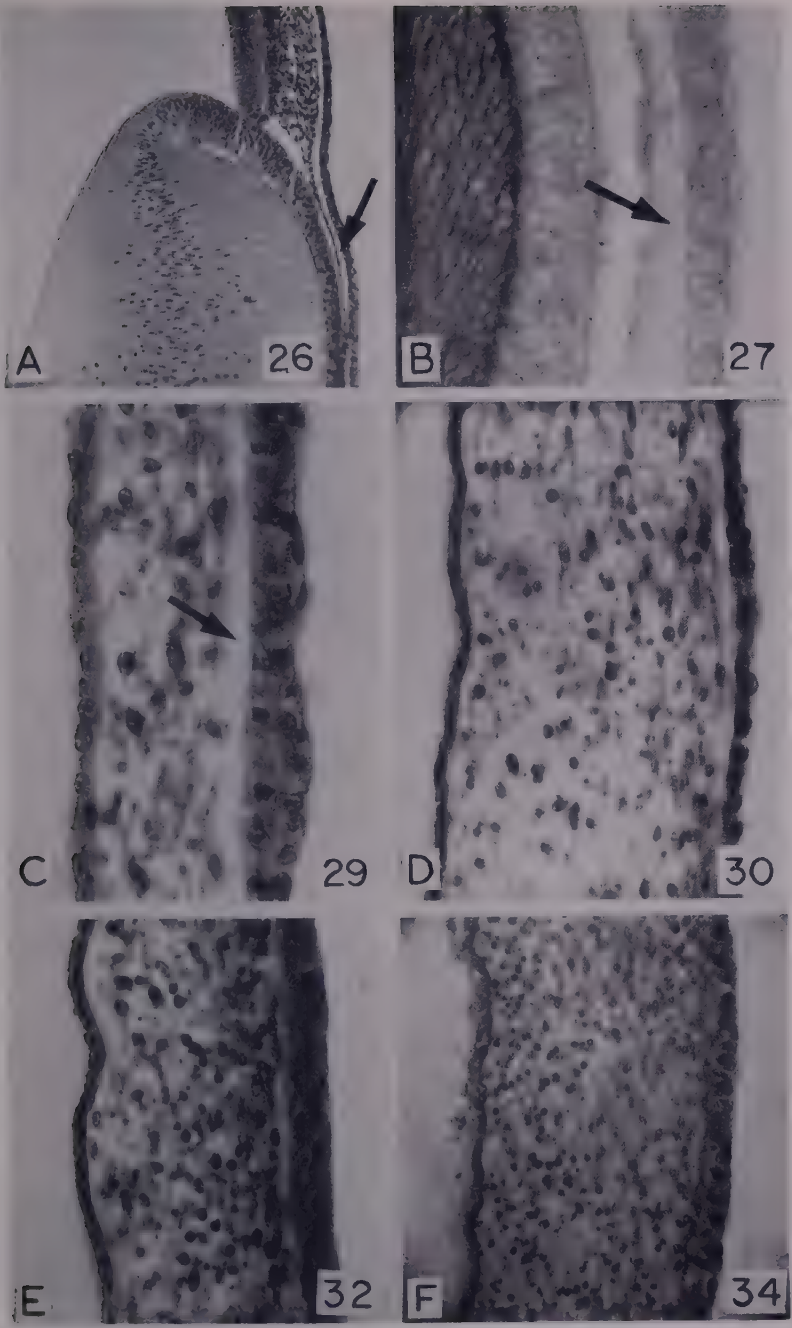

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Plate 1

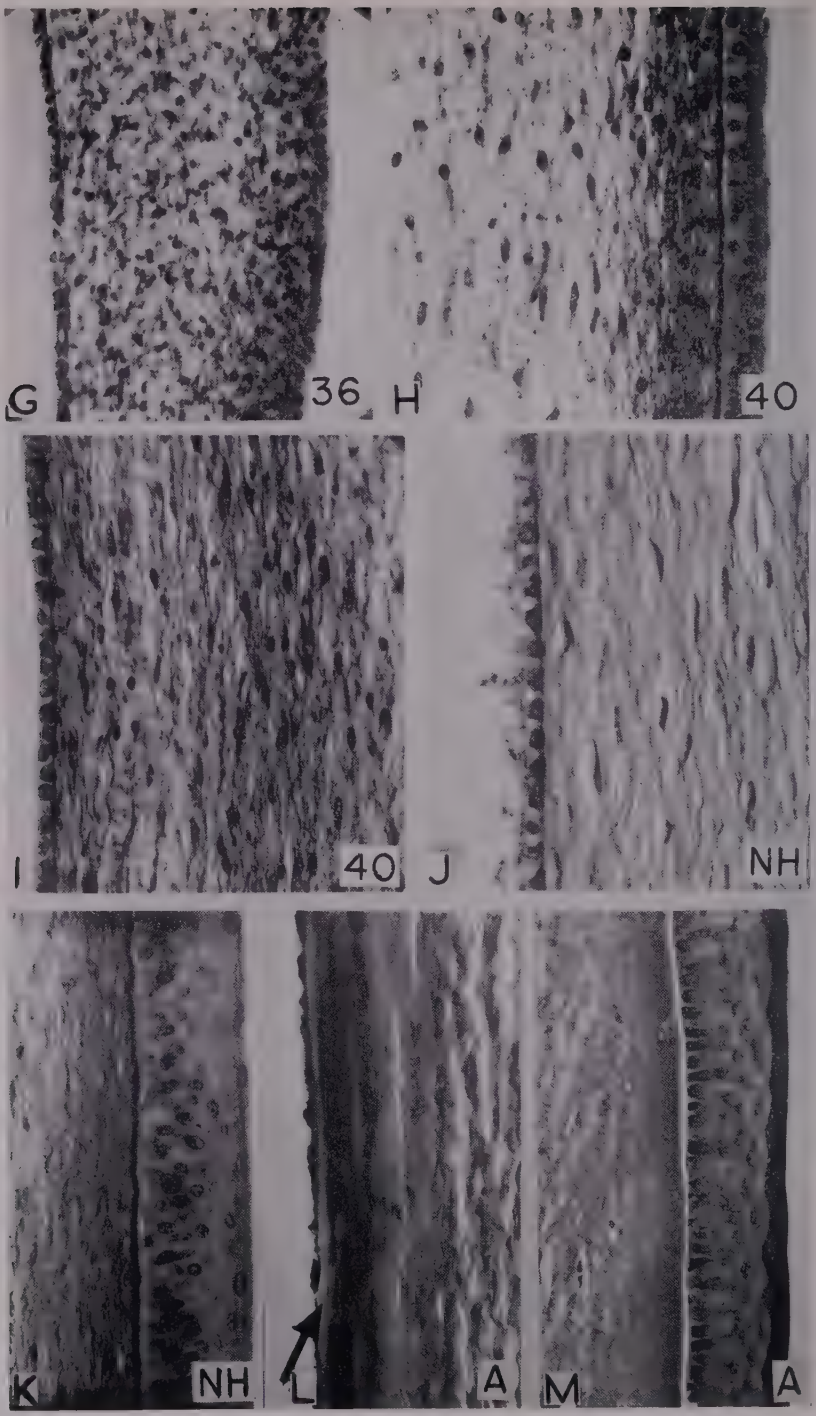

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Plate 2

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EXPLANATION OF PLATES

All the photomicrographs have been passed through a LogEtronics printer. The number on each photograph indicates the stage.

PLATE 1

FIG. A. Stage 26. The cornea consists of an anterior epithelium, a postepithelial layer (arrow), and a mesothelium. × 120.

FIG. B. Stage 27. The anterior epithelium, postepithelial layer (arrow), and mesothelium can be seen here at a higher magnification. The anterior part of the lens is visible deep to the mesothelium. × 400.

FIG. C. Stage 29. The following layers can be seen: the anterior epithelium and its basement membrane, the anterior band of the postepithelial layer (arrow), the *substantia propria*, and the mesothelium. × 533.

FIG. D. Stage 30. Note the basement membranes of the anterior epithelium (right) and the mesothelium (left). The superficial cells of the anterior epithelium had become detached and are not visible in this photograph. × 400.

FIG. E. Stage 32. × 400.

FIG. F. Stage 34. Two thin fibrillar or lamellar bands form the anterior and posterior limits, respectively, of the *substantia propria.* × 267.

PLATE 2

FIG. G. Stage 36. The anterior band is no longer visible. × 400.

FIG. H. Stage 40. The posterior portion of the cornea. Note the basement membrane of the mesothelium. × 400.

FIG. I. Stage 40. The anterior portion of the cornea. Note the anterior limiting lamina. × 400.

FIG. J. Newly hatched (NH). The posterior portion of the cornea. × 533.

FIG. K. Newly hatched (NH). The anterior portion of the cornea. × 400.

FIG. L. Adult (A). The posterior portion of the cornea. The posterior limiting lamina is indicated by an arrow. × 400.

FIG. M. Adult (A). The anterior portion of the cornea. The anterior limiting lamina is not sharply demarcated from the *substantia propria*. The anterior epithelium is artificially separated from the anterior limiting lamina. × 400.

(Manuscript received 19 : xii : 58)

Host-transplant Interactions in Biosynthesis of *Drosophila* Pteridines

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INTRODUCTION

A SERIES of fluorescent pteridines may be revealed and separated by paper chromatography (Hadorn & Mitchell, 1951) from various organs of *Drosophila* in which they appear in high concentrations. Striking differences in the quantities and in the developmental patterns of these compounds are produced by a variety of gene mutations.

While the chemical analysis of the fluorescent substances is rapidly progressing (cf. Forrest & Mitchell, 1955; Viscontini *et al.*, 1955; Viscontini, 1958) the information on the mode and the sites of their biosynthesis is, as yet, extremely scanty. The only well-established reaction involving two of the more abundant pteridines is the enzymatic conversion of 2-amino-4-hydroxy-pteridine (HB_1) to isoxanthopterin (IX) in the presence of the enzyme xanthine dehydrogenase (Forrest, Glassman, & Mitchell, 1956). The importance of this reaction *in vivo* could be demonstrated by experiments with the mutant *rosy* (*ry*) which is devoid of the enzyme (Hadorn & Schwinck, 1956; Hadorn & Graf, 1958; Hadorn, Graf, & Ursprung, 1958). Normalization of IX content in *rosy* eye-disks or testes transplanted into the abdomen of a wild-type host was found to be correlated with a decrease in the concentration of the precursor substance HB_1. Furthermore, injections of HB_1 into wild-type larvae leads to an increase in the IX content of the developing pupae (Graf, Hadorn, & Ursprung, 1959).

The synthesis of the more complex red eye-pigments (drosopterines = DP; cf. Viscontini, Hadorn, & Karrer, 1957) might be assumed to be closely associated with that of IX, because defects and repairs in both fractions appear to be intimately coupled in the mutants *rosy* (Hadorn & Schwinck, 1956) and *maroon-like* (Glassman, Forrest, & Mitchell, 1957). In these genotypes the absence of IX is accompanied by a severe reduction in red pigments, while experimental transfer of eye-tissue to a wild-type host results in concurrent normalization of both deficiencies. It should be noted, however, that other eye-colour mutants like *se* (Hadorn & Ziegler-Günder, 1958) and *Pm* (Ronen, 1957)

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fail to form the drosopterines or contain them in reduced quantities, while IX is present in normal or even increased amounts.

Sepiapteridine (SP) and HB_2 are often increased in genotypes with red-pigment defects (*se*, *ry*, *Pm*) but in other cases (lz^{cl}; Anders, 1955) they may exhibit a corresponding reduction (Hadorn, 1956). The biosynthetic relationship between these two pteridines and the remaining fluorescent compounds is thus completely obscure.

An experimental attack on the differentiation of gene-conditioned substances is rarely possible where gene and endproduct are confined within the limits of the same cell or tissue. If, on the other hand, biochemical differentiation in an organ is governed extrinsically by means of diffusible products, this distance-steering (or non-autonomy) offers an ideal approach for planned interference. The analysis of pteridine relationships should, therefore, be greatly facilitated if the formation of one or several of these compounds was found to be universally non-autonomous.

Indeed, recent work in this laboratory (Hadorn & Ziegler-Günder, 1958; Hadorn, Graf, & Ursprung, 1958) tends to indicate that the level of isoxanthopterin in transplanted eyes and testes is generally and completely host-dependent. While confirming the non-autonomy of isoxanthopterin formation, the present study exploits this phenomenon in order to establish some developmental relationships between IX and the other pteridines. Changes in supply and demand of IX within the host-transplant system are found to be reflected, as a rule, in the concentrations of other pteridine fractions.

MATERIAL AND METHODS

The stocks of *Drosophila melanogaster* utilized were *sepiaoid* (*sed*; 3 64·5 ±), *rosy* (*ry*; 3–51 ±; cf. Hadorn & Schwinck, 1956), *white* (*w*; 1–1·5), and the inbred laboratory stock 'Sevelen'. The strain of *sed* had been backcrossed to Sevelen for nine generations, while *ry* and *w* were not coisogenic with the control stock. In view of the higher isoxanthopterin content of male as compared with female eyes (Hadorn & Ziegler-Günder, 1958) the experiments were restricted to males.

Experimental and control animals of all stocks employed were reared on standard sugar-cornmeal-agar-yeast medium at 25 ± 1° C. The transplantations by means of the method developed by Ephrussi & Beadle (1936) were performed on host and donor larvae during the second half of the third instar. Unless otherwise stated, two eye-disks were transplanted into each host, and the dissections for chromatography were carried out on the 6th day of adult life.

The method employed in one-dimensional chromatography has been described in detail by Ziegler-Günder & Hadorn (1958) and by Hadorn & Graf (1958). Optimal separation by ascending chromatography is achieved with a run of 25 to 27 cm. in propanol-5 per cent. ammonia solution (2:1) if the paper is cut into conical strips below each starting-point and suspended for one hour in the saturated atmosphere of the chromatographic tank before dipping into the solvent.

Hadorn & Graf (1958) summarize the existing information on the composition of the four fractions separated by this procedure. It will therefore suffice to list them briefly, in ascending order of their Rf values:

Group I	DP (drosopterin, neodrosopterin, isodrosopterin; Viscontini *et al.*, 1957).
Group II	XP (isoxanthopterin, xanthopterin, and a substance related to xanthopterin).
Group III	SP (sepiapteridine), for which Forrest & Mitchell (1954) tentatively proposed a structure such as 8-lactyl-7,8 dihydro-2 amino-4-hydroxy-pteridine-6 carbonic acid, and in chromatograms of some *Drosophila* organs, riboflavine (Viscontini *et al.*, 1955).
Group IV	HB (HB_1 = 2-amino-4-hydroxypteridine and HB_2, most probably identical with biopterin).

In group II the fluorometric measurements with the filter systems employed are largely due to xanthopterin. In order to measure isoxanthopterin separately, the chromatograms must be developed in propanol-ammonia 1 per cent. This solvent allows a fair separation of isoxanthopterin and xanthopterin, but the Rf values of SP and HB are so similar with this development that they have to be measured in a single spot.

Two-dimensional chromatography was applied in a few cases. The solvents for the two dimensions were propanol-5 per cent. ammonia (2:1) and butanol-water-acetic acid (20:3:7). In this way it is possible to separate the following compounds: drosopterines, isoxanthopterin, the xanthopterins (xanthopterin and the compound related to it), the SP complex, HB_1, and HB_2.

Owing to development and drying in a darkened room, only negligible amounts of the decomposition product pterine-carbonic acid (Forrest & Mitchell, 1955; Ziegler-Günder, 1956) appeared on the chromatograms. The fluorescence of the spots, traced by long wave UV light, was determined in a fluorometer directly from the dry paper (Hadorn & Kühn, 1953). The primary filter employed has a maximum transmission (m.t.) at 366 mμ, and the secondary filter system consists of two filters with m.t. at 509 and 578 mμ, respectively. Where isoxanthopterin was measured alone, the m.t. of the secondary filters was at 509 and 625 μ.

RESULTS

A. *Effects of inhibited isoxanthopterin synthesis (experiments with* sepiaoid*)*

The mutant *sepiaoid* (*sed*) was chosen for reciprocal eye-transplantations with wild type because of its pronounced deficiency in isoxanthopterin. Table 1 compares the fluorometric values for adult *sed* eyes with those obtained for wild type. In the brown *sed* eyes the drosopterines are much reduced, while the SP and HB fractions are increased about threefold. Two-dimensional chromatography indicates that HB_2 (biopterin) is largely responsible for the rise in the level of the HB group. The XP spot measures slightly more in the mutant. However, visual inspection of the chromatograms by UV light shows that this reading is almost entirely due to xanthopterin, a substance with emerald green fluorescence. In chromatograms of wild type, on the other hand, this spot contains in addition to xanthopterin the isoxanthopterin, which is easily distinguished by its deep violet fluorescence. The level of xanthopterin in *sed* eyes is therefore somewhat higher than indicated in Table 1. Nawa, Sakaguchi, & Taira (1957) were unable to extract xanthine oxidase from *sed* animals. However, this enzyme cannot be completely lacking, since the testes of *sed* males contain a small amount of isoxanthopterin.

TABLE 1

Pteridine groups in sepiaoid (sed) *eyes compared with wild type* (+)

The fluorometric values for *sepiaoid* are expressed as fractions of the mean values for wild type. In this and in all following tables mean values are recorded with ± their standard errors

Genotype	*No. of meas.*	*DP*	*XP*	*SP*	*HB*
+	32	1·00±0·01	1·00±0·02	1·00±0·01	1·00±0·01
sed	22	0·40±0·01	1·18±0·03	3·44±0·13	2·81±0·10

It is possible to circumvent the difficulties arising from the overlap of isoxanthopterin and xanthopterin and to obtain fluorometric readings for pure isoxanthopterin by selecting 50-hour old pupae for chromatographic analysis. Hadorn & Ziegler-Günder (1958) have shown that eye-disks of this stage contain considerable quantities of isoxanthopterin, while the remaining pteridines have not as yet accumulated. The effects of reciprocal eye-transplantations between *sed* and wild type were therefore ascertained, in the first instance, at 50 hours after pupation of the hosts. The results are shown in Table 2. In *sed* hosts neither *sed* nor wild-type eyes are able to form more than a trace amount of isoxanthopterin, but both build up considerable quantities in a normal host. The difference between + in + and *sed* in + is statistically not significant and may not be real.

TABLE 2

Isoxanthopterin content of reciprocal eye transplants between sed *and wild type*

Measured 50 hours after pupation of host in arbitrary fluorometric units

	+ *transplants*			sed *transplants*		
Host	*Fluorometric value*	*No. of meas.*	*No. of eyes*	*Fluorometric value*	*No. of meas.*	*No. of eyes*
+	27·2±5·5	6	36	20·7±2·6	6	36
sed	1·8±0·5	6	36	1·4±0·7	4	20

The isoxanthopterin defect characterizing the *sed* genotype is established as non-autonomous in the data of Table 2. The reaction of the other pteridine fractions to changes in isoxanthopterin synthesis had to be examined in transplants dissected from adult hosts.

Table 3 *a*, *b* summarize the data for normal eye-disks which have developed in the abdomens of wild type and *sed* hosts respectively. Beadle & Ephrussi (1936) observed that the *sed* eye-colour behaves as an autonomous character. Our measurements for the drosopterines are in complete agreement with this statement. Nevertheless, the normal eye is much affected by the *sed* host. The virtual block in isoxanthopterin synthesis is correlated with marked changes in three other pteridine fractions. There is a pronounced increase in xanthopterin, a drop in the sepiapteridine group and an enhanced accumulation of HB. Table 3*b* is based on two-dimensional chromatograms. In spite of the large variance obtained with this method, it gives some additional information: the net increase in xanthopterin in the *sed* host is seen to be about threefold. Thus, while the *sepiaoid* mutant possesses only a little more of this substance than wild type (cf. Table 1) it is produced in very large quantities by the normal eye in the environment of a *sepiaoid* host. Table 3*b* also indicates that the rise in the HB fraction involves HB_1 (the known precursor of isoxanthopterin) while HB_2 appears to be decreased.

TABLE 3*a*

The influence of sepiaoid *hosts on wild-type eye-transplants*

DP: the mean fluorometric reading for + in + is fixed as unity. XP, SP, and HB: for each spot, the readings of these groups were expressed as fractions of DP readings. The mean ratios determined for + in + were fixed as unity

+eye-disks in	*DP*	*XP*	*SP*	*HB*	*No. of meas.*	*No. of transpl.*
+ hosts	1·00±0·03	1·00±0·06	1·00±0·05	1·00±0·06	18	36
sed hosts	0·98±0·04	1·65±0·10	0·74±0·04	1·31±0·07	20	40

TABLE 3*b*

The influence of sepiaoid *hosts on wild-type eye-transplants (from two-dimensional chromatograms)*

All fluorometric values are recorded as percentage of DP readings of corresponding chromatograms

+eye-disks in	*IX*	*XP*	*SP*	HB_1	HB_2	*No. meas.*	*No. transpl.*
+hosts	52·5±4·2	25·3±3·9	27·4±7·0	9·6±1·6	23·9±3·0	4	40
sed hosts	17·1*±2·1	74·9±7·8	16·1±0·8	21·7±5·4	17·2±2·4	4	27

* No visible violet fluorescence in this spot.

The impact of a normal environment on the fluorescent substances of a *sepiaoid* eye is shown in Table 4. The negative correlation between isoxanthopterin synthesis and the levels of xanthopterin and HB which was observed in normal eye-disks (Table 3 *a*, *b*) is again manifest. However, the *sed* eye fails to show any positive correlation between isoxanthopterin and the SP complex.

TABLE 4

The influence of wild-type hosts on sepiaoid *eye-transplants*

DP: the mean fluorometric reading for *sed* in *sed* is fixed as unity. XP, SP, and HB: for each spot, the readings of these groups were expressed as fractions of DP readings. The mean ratios determined for *sed* in *sed* were fixed as unity

sed *eye-disks in*	*DP*	*XP*	*SP*	*HB*	*No. of meas.*	*No. of transpl.*
sed hosts	1·00±0·05	1·00±0·03	1·00±0·02	1·00±0·05	23	46
+ hosts	0·96±0·04	0·87±0·03	0·96±0·02	0·82±0·03	30	60

These experiments demonstrate that the wild-type organism produces an agent which normalizes the isoxanthopterin level of *sepiaoid* implants without affecting their content in red pigments. It is possible that the material passed by the host to the implant might be identical with the ry^{-} agent postulated by Hadorn & Schwinck (1956), though the latter supplements the defects in both the isoxanthopterin and the drosopterin fractions of *rosy*. Since wild-type fat-bodies were found (Hadorn & Schwinck, 1956) to constitute one of the sources of this agent, their effects on the isoxanthopterin metabolism of *sed* males were tested. Fat-bodies of the stock '*altered ratio*' (*a.r.*) were also injected into *sed* hosts. The *a.r.* stock exhibits an excess of isoxanthopterin in addition to other changes in pteridine ratios as compared with wild type (Goldschmidt, 1958).

The testis of *sed* in which the isoxanthopterin content is reduced to about one-twelfth of wild type served as a test object. The injections produced a small but significant rise in the IX level of the host testes. There was also an increase in the SP fraction of the experimental testes. It is true that in fat-body injections no more than one-eighth to one-sixth of the total fat-body content of the donor can be transferred. But this dosage problem can hardly account for the large scatter observed in the present experiment as well as in that of Hadorn & Schwinck (1956), in which the effect was more drastic.

B. *Experimental separation between the drosopterin deficiency and the isoxanthopterin block of the* rosy *phenotype*

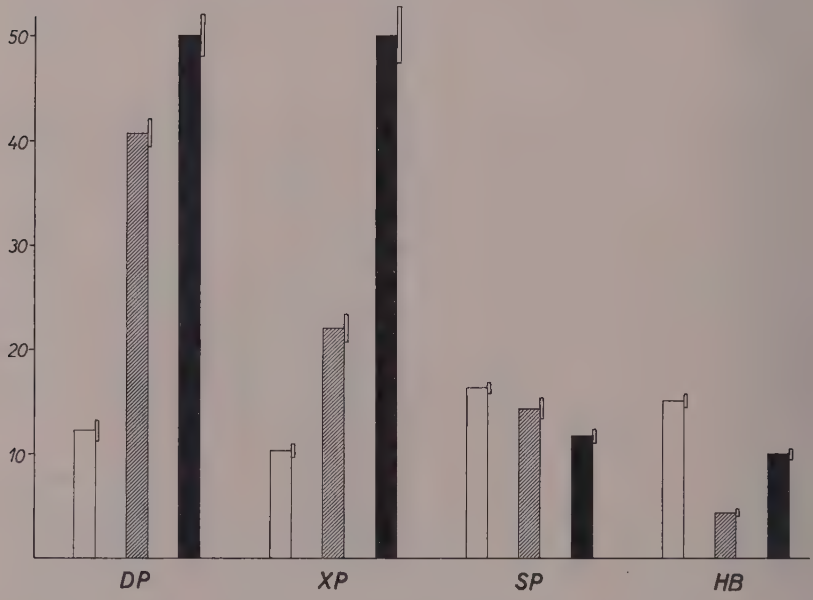
While Hadorn & Schwinck (1956) had demonstrated that a normal host supplies the material required by *rosy* eyes for normalization of isoxanthopterin and of the red pigments, Hadorn & Graf (1958) investigated the repercussions of this repair on other pteridine fractions. The abnormally high SP and HB concentrations characterizing *rosy* were observed to drop in a wild environment. Since drosopterines and isoxanthopterin showed correlated changes in this experimental situation, it could not be decided whether they were jointly or separately responsible for the other shifts in the pteridine pattern.

TABLE 5

Relative pteridine fractions in ry *transplants in normal and in* sepiaoid *hosts*

For each spot, XP, SP, and HB readings were recorded as fractions of DP readings. The mean ratios determined for *ry* in + were fixed as unity

ry *eye-disks in*	*XP*	*SP*	*HB*	*No. of meas.*	*No. of transpl.*
+ hosts	1·00±0·04	1·00±0·05	1·00±0·05	14	28
sed hosts	1·86±0·11	0·71±0·03	2·04±0·12	15	30



TEXT-FIG. 1. Pteridine fractions in *ry* eye-transplants in three different hosts. White columns: in *ry* hosts (based on 20 transplants). Cross-hatched columns: in + hosts (based on 34 transplants). Black columns: in *sed* hosts (based on 30 transplants). The heights of the columns correspond to arbitrary fluorometric values for the four pteridine fractions. The bars attached to the columns indicate the extent of the standard errors of the means.

We have shown that drosopterin differentiation of a normal eye rudiment is not impeded in a *sepiaoid* host, while isoxanthopterin metabolism is severely disturbed. Hence this host should provide suitable conditions for the experimental separation of the two pteridine defects in *rosy* metabolism. The results appear in Table 5 and Text-fig. 1. It is seen that the *sed* hosts have corrected the drosopterin defect of *ry* eyes. *Rosy* transplants in *sed* hosts had formed even somewhat more red pigment than those developed in wild type. But this difference should be regarded with caution, since it may be due to discrepancies in developmental rates between the two host strains. The XP fraction of *ry* eyes grown in *sed* revealed no isoxanthopterin on inspection under UV light. There was instead a very considerable accumulation of emerald xanthopterin, similar to that observed in + eyes in the same host (cf. Table 3). The SP group is much more reduced in the *sed* host than in the normal host. This might indicate a direct link between one of the SP components and isoxanthopterin metabolism. With regard to HB, the *sed* environment determines a level which is intermediate between the excess in a *rosy* host and the normalized concentration in +. The drop in this fraction in a *sed* host is probably due to HB_2. It is instructive to compare Table 5 with Table 3 and to observe the parallels in the reactions of *rosy* and + eyes in the *sed* hosts.

The correlation obtained in previous experiments with *rosy* (Hadorn & Graf, 1958) for the adjustment in drosopterines and in isoxanthopterin had suggested a close association between the synthesis of these two pteridine classes. The present results do not support this assumption. In the hierarchy of the *rosy* syndrome, at all events, the two pteridine defects must occupy widely differing ranks.

C. *Effect of excretory depletion in pteridines of host environment*

Inherited synthetic blocks are not the only agents affecting the pteridine pattern of adult flies. The genotypes *w* and *bw* form considerable quantities of isoxanthopterin and of the SP and HB groups during pupal life (Hadorn, 1954; Hadorn & Ziegler-Günder, 1958). These pteridines are subsequently eliminated by the excretory system, especially with the meconia (Hadorn & Kürsteiner, 1955). The general correspondence between isoxanthopterin levels of hosts and transplants (cf. sections A and B) which becomes manifest in the early pupa (section A) establishes the dominance of the host in setting the rate of synthetic activity. It seemed important to find out whether pteridine retention in the transplants is also linked up with storage or depletion in the host.

The pteridine pattern was therefore examined in wild-type eyes developed in the abdomens of *w* hosts (Table 6). The transplants contained, on the average, two-thirds of the drosopterin quantity which is found in an eye *in situ*. This is as much as a transplant will usually develop in a normal host (Hadorn & Schwinck, 1956; Hadorn, 1957). Thus the red pigments behave autonomously in this experiment, as claimed by Beadle & Ephrussi (1936) from inspection of the eye-colour. We are here exclusively concerned with the other pteridines.

The relative concentrations of these substances were found to be essentially normal although the SP fraction is more abundant than in wild-type controls. Similarly, *w* transplants in normal hosts had followed their intrinsic excretory pattern and only non-measurable traces of pteridines were found to be retained by them.

TABLE 6

The influence of white *eyed hosts* (w) *on pteridine fractions of normal eye-transplants*

DP: the mean fluorometric reading for + controls is fixed as unity. XP, SP, and HB: for each spot, the readings of these groups were expressed as fractions of DP readings. The mean ratios determined for + controls were fixed as unity

	DP	*XP*	*SP*	*HB*	*No. meas.*	*No. transpl.*
+controls	1·00±0·02	1·00±0·03	1·00±0·04	1·00±0·04	10	. .
+eyes in *w* hosts	0·68±0·04	1·06±0·04	1·27±0·04	1·07±0·05	9	18

It may be concluded that pteridine levels of hosts and transplants are independent during later pupal and imaginal life. The host influence via diffusible products is restricted to the phase of pteridine synthesis. Pteridine excretion in the *w* genotype appears to be cell-specific rather than governed by gradients pervading the whole organism.

D. *Shifts in isoxanthopterin requirement within the host-transplant system*

The non-autonomy of isoxanthopterin synthesis in eyes and in testes (Hadorn, Graf, & Ursprung, 1958) implies the production by the host of a prerequisite compound for this reaction. It is tempting to assume that this substance may be the enzyme xanthine-dehydrogenase (cf. Forrest, Glassman, & Mitchell, 1956), although, in this case, its penetration into the transplants would not be easy to visualize.

The main concentration of isoxanthopterin occurs in eyes and testes. A smaller quantity is present in the Malpighian tubules. These organs may be assumed to compete for the host-supplied 'agent'. It is possible to increase the demand for this substance in eye-tissues by charging a host with a maximum of eye-transplants. Hadorn (1957) has shown that a host is able to support up to eight implanted eye-disks. The drosopterin content in each transplant of such a group was similar to that of an eye transplanted singly. The red pigments of host eyes were also undiminished.

It was decided to study the remaining pteridines in such an overloaded system. Five or six wild type eye-disks were transplanted into normal larvae. The changes in the eyes of hosts and transplants which appear in Table 7 *a*, *b* came as a surprise. A net increase in isoxanthopterin content of host eyes and transplants is obtained. The concentration of the SP + HB fraction is also higher than in the controls. Two-dimensional chromatography indicates (Table 7*b*) that the SP fraction contributes largely to this effect.

It is probably wrong to attribute this excess in eye isoxanthopterin throughout the system to a stimulated production of the 'agent' by the host. The key to the phenomenon is to be found in the behaviour of the host testes. Hadorn, Graf, & Ursprung (1958) observed that the isoxanthopterin content of a testis is proportional to its size. A testis which fails to establish contact with a vas deferens remains relatively small and rounded and accumulates only half of the isoxanthopterin quantity that is present in the normally attached and coiled organ. The testes of host males harbouring six eye-transplants exhibit invariably some slight compression. In some cases they may be severely deformed. The pteridine inventory of normally coiled and of damaged host testes is presented in Table 8. The isoxanthopterin of damaged testes is reduced. The trend in the normally coiled testes is similar.

TABLE 7*a*

The influence of increased isoxanthopterin supply on host eyes and transplants (from one-dimensional chromatograms)

5–6 wild-type eyes were transplanted into wild-type hosts.
T_6: transplants from groups of 6
T_5: transplants from groups of 5
H_6: host with 6 transplants
H_5: host with 5 transplants

For each spot, XP, SP, and HB readings were recorded as fractions of DP readings. The mean fractions for + controls are fixed as unity

	IX	*SP+HB*	*No. of meas.*	*No. of transpl.*
T_6	1·51±0·08	1·22±0·04	11	23
T_5	1·34±0·07	1·19±0·08	7	21
H_6	1·24±0·09	1·07±0·05	6	..
H_5	1·19±0·05	1·07±0·04	5	..
+contr.	1·00±0·03	1·00±0·03	15	..

TABLE 7*b*

The influence of increased isoxanthopterin supply on host eyes and transplants (from two-dimensional chromatograms)

For explanation cf. Table 7*a*. Pteridine fractions for each chromatogram are recorded in percentage of DP readings

	IX	*XP*	*SP*	*HB*$_1$	*HB*$_2$	*No. of meas.*	*No. transpl.*
T_6 and T_5	94·0±7·3	21·8±2·4	41·0±3·2	10·7±3·2	19·8±2·2	7	87
H_6 and H_5	69·8±5·0	19·8±1·8	44·9±8·7	10·4±3·9	18·2±2·2	4	..
+controls	47·0±3·3	20·4±4·2	33·0±2·4	10·5±3·6	18·2±2·3	3	..

The extent of the damage incurred by the compressed testes may be estimated from the fact that their colour was orange instead of yellow owing to the accumulation of a reddish-brown pigment. Chromatography indicates that these pigments are not drosopterines. They probably belong to the class of ommochromes. This reaction of the testes to trauma is in conformance with the findings of Ursprung, Graf, & Anders (1958) who observed ommochromes in Malpighian tubules injured by UV irradiation. In view of this serious disturbance we should refrain from basing any inferences on the abnormal pteridine ratios of the compressed testes (cf. Table 7).

TABLE 8

Influence of compression and damage on pteridine content of host testes

For explanation of experiment cf. Table 7*a*. IX: the mean fluorometric reading for unoperated controls is fixed as unity. XP, SP, HB: for each spot, the readings of these groups were expressed as fractions of DP readings. The mean ratios determined for unoperated controls were fixed as unity

	IX	*XP*	*SP*	*HB*	*N*
Controls	1·00±0·03	1·00±0·07	1·00±0·04	1·00±0·03	11
Host testes slightly damaged	0·91±0·06	1·40±0·12	1·25±0·11	1·12±0·10	11
Host testes severely damaged	0·73±0·06	1·48±0·17	1·20±0·06	1·17±0·11	5

It is obvious, however, that these testes were unable to claim their full share of the prerequisites for isoxanthopterin synthesis. The host eyes as well as the transplants were able to profit from this fact. The advantage of transplants over host eyes in this competition must be connected with their abdominal position in the vicinity of the disturbed testes. A direct supply of HB_1 from testes to transplants may be involved. The increased isoxanthopterin metabolism in the eyes is associated with an increase in the SP group.

DISCUSSION

There are numerous inherited differences in isoxanthopterin levels of *D. melanogaster*. Males possess more of this substance than females (Hadorn & Ziegler-Günder, 1958). Major genes give rise to changes and sometimes to blocks in isoxanthopterin synthesis (Hadorn & Mitchell, 1951; Hadorn & Schwinck, 1956). Wild-type stocks exhibit much variation with regard to this character (Robertson & Forrest, 1957). It is doubtlessly dependent on a large number of genes.

Nevertheless, when two such different genotypes are combined in a transplantation experiment, the outcome appears to be uniform; the genotype of the host determines the isoxanthopterin content of the transplant. For the mutant *rosy* this conclusion was reached by Hadorn & Schwinck (1956) and by Hadorn & Graf (1958). Non-autonomy of the difference between males and females could also be demonstrated (Hadorn & Ziegler-Günder, 1958; Hadorn, Graf, & Ursprung, 1958). The present study establishes non-autonomy for the severe defect in isoxanthopterin which characterizes the mutant *sepiaoid*. In all these cases the dominance of the host in setting the rate for isoxanthopterin synthesis might be ascribed to a single diffusible 'agent' which is supplied to the transplant primordia in gene-conditioned quantities.

It could be shown that non-autonomous changes in the rate of isoxanthopterin formation are correlated with shifts in at least three other pteridine fractions. Not only the known precursor of isoxanthopterin (HB_1), but also the 'xanthopterin' and SP groups, are affected. The levels of all these pteridines are therefore host-dependent, although it may be correct to consider isoxanthopterin synthesis as the primary non-autonomous process.

TABLE 9

Correlated changes negative (—) or positive (+) of pteridine groups

The behaviour of the XP group should be interpreted with caution. It may reflect peculiarities of the *sed* genotype

1. *Correlations with isoxanthopterin formation*

Type of experiment	*Table*	*XP*	*SP*	*HB*
+ in *sed* compared with + in +	3 *a*, *b*	—	+	—
sed in + ,, ,, *sed* in *sed*	4	—	?	—
ry in + ,, ,, *ry* in *sed*	5 Text-fig. 1	—	+	—
Damage to testes increases IX synthesis in transplants and host eyes	7 *b*		+	

2. *Correlations with drosopterin formation*

Type of experiment	*Table*	*XP*	*SP*	*HB*
ry in *sed* compared with *ry* in *ry*	Text-fig. 1	+	—	—

Table 9 summarizes some of the correlations between these non-autonomous pteridines, as observed in the present experiments. It presents repeated evidence for an increase in the SP fraction with rising IX level and for an accumulation of xanthopterin when IX synthesis is inhibited. This latter phenomenon is so pronounced that it cannot be disregarded as an artefact of alkaline chromatography (Ziegler-Günder & Hadorn, 1958). However, it should be stressed that in *rosy* hosts (Hadorn & Graf, 1958) a normal transplant which is blocked in its IX production exhibits no increase in its XP fraction. This storage may be peculiar to *sed* metabolism.

A stepwise repair of the two pteridine defects in the mutant *rosy* has contributed some information on drosopterin formation. A *sed* host enables *ry* transplants to build up a normal quantity of red pigments without correcting their lack of isoxanthopterin. It appears that components of both the SP and the HB group participate in the formation of drosopterines. This experimental separation of two *rosy* phenes is not easily compatible with the concept of a single *ry*$^{+}$ agent, affecting DP and IX directly and jointly.

Reduction in the size of the testes due to pressure exerted by multiple eye transplants induces increased isoxanthopterin storage in both the foreign and the host eye-rudiments. We infer that under normal conditions the different organs compete for a limited supply of isoxanthopterin. In this experiment again, increased IX formation is coupled with the storage of an SP component.

The elimination of isoxanthopterin and of other pteridines in the genotype *w* (Hadorn, 1954) is a cell-specific process. It is not regulated by the composition of the haemolymph or by the activity of the Malpighian tubules. A *w* eye excretes its pteridines in a wild-type environment and a normal eye is able to maintain its fluorescent substances in a *w* host. Thus, pteridine retention, in contrast to pteridine synthesis, involves no communication between host and transplant.

SUMMARY

1. The isoxanthopterin deficiency of the mutant *sepiaoid* is a non-autonomous character.

2. Non-autonomous changes in the level of isoxanthopterin are correlated with changes in three other pteridine fractions.

3. A *sepiaoid* host is able to normalize the red pigments in implanted eyes of the mutant *rosy*, but does not repair its isoxanthopterin defect. The reaction of other pteridines to drosopterin formation can thus be separately observed.

4. While isoxanthopterin synthesis may generally be host dependent, its retention in the imago is autonomous. Wild-type eyes maintain their pteridine levels in a *white* host that excretes its own fluorescent materials.

5. Compression of the testis by numerous eye transplants diminishes its isoxanthopterin storage. The surplus of diffusible precursors leads to increased deposition in host eyes and eye-transplants.

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Recherches sur les malformations déterminées par le refroidissement temporaire de l'œuf de poule et compatibles avec la vie des Poussins

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NOUS avons signalé dans un travail antérieur (Ancel, 1958) portant sur 699 œufs que le refroidissement de l'œuf de poule à différentes périodes de l'incubation pendant deux ou trois jours donnait naissance à des malformations des paupières, du bec et des membres. Un petit nombre d'embryons avaient été prélevés seulement après le 17ème jour de l'incubation. Dans le présent travail, nous avons cherché à savoir si certaines des malformations ainsi obtenues étaient compatibles avec la vie des poussins.

TECHNIQUE

Les œufs de Leghorn sont retirés de la couveuse réglée à 38° et placés pendant 2 jours dans un local dont la température a varié de 17 à 22°. Les expositions à ce froid relatif ont été faites, suivant les expériences, du 4ème au 6ème jour, du 5ème au 7ème, du 6ème au 8ème, du 7ème au 9ème et du 8ème au 10ème jour de l'incubation.

Après 72 heures d'incubation, une fenêtre ronde de 1,5 cm. de diamètre environ a été ouverte dans la coquille après prélèvement de 2 c.c. d'albumine par un petit trou fait dans le bout pointu de l'œuf. La fenêtre est obturée par une lamelle de ruban adhésif au travers de laquelle les embryons sont examinés au moment de la mise au froid. Les morts et les anormaux sont éliminés de l'expérience. Les autres sont examinés tous les matins à partir du lendemain de la remise en couveuse et les morts sont étudiés au point de vue tératogène. Au 22ème jour de l'incubation, on retire la lamelle de ruban adhésif pour faciliter l'éclosion des embryons porteurs de malformations du bec rendant difficile le bêchage de la coquille.

Les résultats obtenus ont été résumés dans le tableau.

CONSIDÉRATION SUR LES RÉSULTATS

Fréquence des malformations

Dans l'ensemble, que les embryons soient morts avant ou après l'éclosion, nous avons retrouvé les malformations que nous avions obtenues dans notre

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précédent travail mais dans des proportions différentes. Comme dans cette première recherche, l'ablépharie ne s'y est montrée qu'après le refroidissement du 4ème au 6ème jour, du 5ème au 7ème et du 6ème au 8ème, ainsi que le buphtalmos que nous avions en outre observé après refroidissement du 7ème au 9ème jour.

Le bec de lièvre (arrêt de développement du bourgeon maxillaire supérieur) n'a été observé dans les deux travaux qu'après refroidissement du 4ème au 6ème jour et du 5ème au 7ème.

Les plus fortes proportions de malformations des membres ont comme précédemment été trouvées dans les expériences de refroidissement du 7ème au 9ème et du 8ème au 10ème jour.

Ainsi se confirme que le stade auquel débute le refroidissement a une forte influence sur la variété de malformation obtenue.

Comme différence entre les résultats obtenus dans nos deux travaux, nous signalerons l'atrophie du bec observée après refroidissement du 6ème au 8ème et du 7ème au 9ème jour comme précédemment, mais en outre du 5ème au 7ème et du 8ème au 10ème en très faibles proportions il est vrai (2,1 et 1,7) mais sur un ensemble de 393 embryons, tandis que ce résultat n'avait pas été obtenu après les mêmes expériences portant sur 699 embryons.

Résultats relatifs aux éclosions

La fréquence des éclosions a progressivement diminué au fur et à mesure que les œufs étaient exposés au froid à un stade plus avancé (de 13,5 % à 3,5 %). Le tableau montre aussi que certains œufs éclosent avec des embryons porteurs de malformations, mais dans de faibles proportions par rapport au nombre des œufs mis en expérience (5,2 au maximum).

Le tableau montre que l'ablépharie localisée est la malformation qui se rencontre chez presque tous les porteurs de malformations après l'éclosion à la suite du refroidissement du 4ème au 6ème, du 5ème au 7ème et du 6ème au 8ème jour de l'incubation, tandis qu'après le refroidissement du 7ème au 9ème jour, l'ablépharie n'a plus été constatée, les malformations des membres l'ont remplacée ainsi que l'atrophie du bec.

Notre but principal étant de savoir avec quelles variétés de malformations les embryons soumis au froid pouvaient éclore, nous n'avons essayé d'élever que 4 de ces poussins éclos avec des malformations, 3 d'entre eux étaient porteurs d'ablépharie et 1 de malformations des membres. Nous avons été amenés à en tuer 3 pour cause de déficience ne leur permettant plus de se tenir droits sur leurs pattes ou pour cétité. Notre désir était de savoir si le refroidissement de l'œuf était capable de permettre à des poussins ne différant des normaux que par une malformation, d'atteindre l'âge adulte. Une réponse affirmative a été donnée par un poussin qui est actuellement un coq âgé de 12 mois et qui ne diffère des normaux que par ablépharie symétriquement localisée à la paupière supérieure.

Ce résultat semble ne pouvoir être obtenu que sur un petit nombre d'embryons soumis au froid. Les meilleures conditions paraissent être le refroidisse-

Tableau résumant les effets létaux et tératogènes du séjour des œufs au froid pendant 48 heures entre le quatrième et le dixième jour de l'incubation

Jours de froid	*Nombre des embryons en expérience*	*Morts 24 heures après remise en couveuse*	*Vivants au 10ème jour*	*Vivants au 20ème jour*	*Embryons porteurs de malformations*	*Ablépharie*	*Buphtalmos*	*Bec de lièvre*	*Malformations des membres*	*Atrophie du bec*	*Embryons éclos total*	*Embryons éclos malformés*	*Embryons éclos abléphariques*
		%	%	%	%	%	%	%	%	%	%	%	%
4 à 6	133	12	71,6	49,3	30,8	27,8	9,8	12	3,7	0	13,5	5,2	4,5
5 à 7	95	35,7	50	29,4	21	15,8	4,2	2,1	3,1	2,1	10,5	4,2	3,1
6 à 8	56	39,2	42,8	16	16	8,9	7,1	0	3,5	1,7	8,8	1,7	1,7
7 à 9	53	30,1	69,8	20,2	18,8	0	0	0	9,4	7,5	3,7	3,7	0
8 à 10	56	46,4	—	17,8	14,2	0	0	0	10,7	1,7	3,5	0	0
	393												

Les chiffres des deux premières colonnes indiquent les 5 différentes périodes où les œufs ont été refroidis et leur nombre pour chaque période. Dans les autres colonnes, ils donnent des pourcentages.

ment du 4ème au 6ème jour puisque ce sont celles dans lesquelles la proportion des éclosions est la plus grande et aussi celle des embryons porteurs de malformations et celle des embryons ablépharиques. Outre les cas où la vitalité du poussin ne lui permet pas de se tenir droit sur ses pattes le lendemain de l'éclosion, les causes d'échec dans l'élevage sont l'existence d'un bec croisé, dû ordinairement à la présence d'un bec de lièvre unilatéral, ou les cas d'ablépharie dans lesquels la commissure postérieure des deux paupières fait entièrement défaut. Dans ces cas, en effet, une secrétion s'accumule sur l'œil auquel adhère plus ou moins du duvet qui aveugle le poussin; il en est de même du buphtalmos nettement saillant qui est cause de cécité et qui finit par se rompre ouvrant la chambre antérieure de l'œil.

DISCUSSION

L'existence du poussin devenu adulte ablépharique nous permet d'affirmer qu'un agent physique intervenant au cours du développement embryonnaire avant l'éclosion peut réaliser une malformation dont un adulte est porteur, parce que la malformation observée dans ces conditions ne se rencontre pas à l'état spontané.

Au cours de recherches de tératogénèse expérimentale faites par de nombreux auteurs, à l'aide d'agents physiques ou chimiques, les embryons ont été habituellement prélevés avant le 18ème jour de l'incubation, le but des recherches étant de savoir quelles variétés de malformations pouvaient être obtenues par différents agents. Au cours de ces expériences, l'existence d'une malformation chez un poussin après l'éclosion n'aurait pu être considérée comme déterminée par l'agent tératogène utilisé, parce qu'il aurait pu s'agir d'un cas de malformation d'origine génétique.

Au cours de recherches de chimiotératogénèse, nous avons observé par exemple que le méthylarsinate de soude produisait l'anourie, et nous avons élevé un anoure jusqu'au 8ème jour après l'éclosion, bien que se manifestent des signes de déficience générale qui nous ont amené à mettre fin à cet élevage. L'anourie spontanée se rencontrant dans une proportion supérieure à 1 pour cent, aucune preuve ne peut être donnée que, dans ce cas, la malformation était due à la substance chimique utilisée.

RÉSUMÉ

Le refroidissement temporaire de l'œuf de poule Leghorn à divers stades pendant deux jours, au cours de l'incubation, permet d'obtenir des malformations observables après l'éclosion. Un coq adulte qui, à l'état embryonnaire, avait subi un refroidissement du 4ème au 6ème jour, a été obtenu porteur d'ablépharie localisée bilatérale.

SUMMARY

Cooling of the eggs of Leghorn hens for 2 days during incubation has produced malformations detectable after hatching. An adult cock with a bilateral localized ablephary was obtained after cooling during the 4th to the 6th days of embryonic life.

TRAVAIL CITÉ

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The Effects of the Chelating Agent Ethylenediamine Tetra-acetic Acid on Cell Adhesion in the Slime Mould *Dictyostelium discoideum*

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WITH ONE PLATE

INTRODUCTION

CERTAIN properties of living cells appear to depend primarily on changes at, or characteristics of, the cell surface or plasma membrane. Among these 'surface-linked' phenomena are to be classed adhesion of cells to their neighbours or substratum, pseudopodial activity and plasma membrane stability, and, frequently, cell and tissue movements. (Others which might be mentioned, such as pinocytosis, trans-membrane movements of substances, and vacoule formation, will not be considered here.) Attempts to examine these properties in terms of chemical mechanisms have not been notably successful, owing in part to the fact that the experimental material has traditionally been the tissues or embryos of metazoan forms. Thus, investigators have worked with heterogeneous and often constantly changing populations of cells, from which individual cells could be obtained only by the use of more or less deleterious methods such as mechanical separation or treatment with disaggregating agents. It appears that a simpler system is needed, a system of cells with definite and reproducible adhesive and migratory characteristics, undergoing no major histogenetic changes, and easily isolated and maintained in the laboratory.

These requirements are largely met by at least one organism available to the experimenter, namely the cellular slime moulds (Acrasieae). During its life cycle this primitive form goes through a vegetative stage in which it exists as individual amoeboid cells, the myxamoebae, which wander about freely on the substratum feeding on bacteria. Under the influence of acrasin, these cells soon begin migrating toward aggregation centres where they clump together to form a slug-like pseudoplasmodium (Raper, 1935; Bonner, 1947). During this aggregative phase, according to Shaffer (1957), the surface characteristics of the cells alter so that they become 'sticky', forming pseudopodial attachments to one another. In those combinations that have been studied, adhesion has proved to be species

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specific (Raper & Thom, 1941; Bonner & Adams, 1958), and this specificity is reported to be associated with differences in surface antigens (Gregg, 1956; Gregg & Trygstad, 1958). Only after aggregation into the pseudoplasmodium does recognizable histo-differentiation occur, accompanying the onset of the fruiting stage (Bonner, Chiquoine, & Kolderie, 1955). With such an organism it can be seen that alterations in surface characteristics associated with the change from free-living vegetative cells to adhesive aggregating cells can be studied with appropriate experimental techniques.

One of the characteristics of membrane-linked phenomena which seems common to many metazoan cells is their response to the removal of calcium. Various degrees of calcium (and/or magnesium) depletion can lead to limited cell dissociation resulting in abnormal morphogenetic movements of embryonic layers (Okazaki, 1956; DeHaan, 1958), instability of the cell surface with violent pseudopodial activity and surface blebbing (Dornfeld & Owczarzak, 1958), and, finally, to complete disaggregation (Anderson, 1953; Zwilling, 1954; Feldman, 1955; Steinberg, 1958).

It is the purpose of the present work to determine whether or not slime mould cells react to calcium removal in a manner analogous to those of metazoan tissues. If not, further study of membrane-linked phenomena in these cells as prototypes would seem unwise.

MATERIALS AND METHODS

All the experiments to be described were performed on members of the species *Dictyostelium discoideum* Raper, strain V–12. Stock cultures were carried on the following agar medium (PGT 1): peptone, 1 gm.; dextrose, 1 gm.; NaCl, 0·6 gm.; KCl, 0·75 gm.; $CaCl_2$, 0·3 gm.; $MgSO_4$, 0·2 gm.; bacto-agar, 20 gm.; tris(hydroxymethyl)aminomethane, 4·84 gm.; distilled water, 1,000 ml. This mixture was brought to a pH of 6·6–6·8 with normal NaOH and autoclaved for 20 minutes at 15 lb. pressure. Cultures grown on PGT 1 proved consistently better than those grown on Bonner's agar medium (Bonner, 1947) or on a medium similar to PGT 1 but buffered with 40 mM phosphate instead of tris. Extensive trials were run to demonstrate that tris buffer at concentrations up to 0·1 M has no observable deleterious or inhibitory effect on the development of this species. On PGT 1, cultures grow to about the same density and at a similar rate as those grown on hay infusion agar (Raper, 1951). More luxuriant growths can be obtained with PGT 5 and PGT 10, in which the peptone and glucose are each increased to 5 and 10 gm. per litre, respectively, all other components remaining the same as in PGT 1. Cultures were transferred by inoculating spores on to an agar plate freshly streaked with a suspension of *Escherichia coli*.

Hanging-film chambers were prepared by cementing an alcohol-rinsed coverslip to a polyethylene ring 5 mm. deep, cut from polyethylene tubing half an inch in diameter. After a cell suspension had been added and time allowed for the cells to settle on to the coverslip the upper edge of the ring was greased with petrolatum and a microscope slide was pressed on to it, forming a floor to the chamber. The chamber was then turned coverslip side up with the cells adhering to its under surface so that they were accessible to observation at high magnifications. Normal aggregation and fruiting will occur in these circumstances.

Cell suspensions were prepared by washing a plate of vegetative myxamoebae into a centrifuge tube with 10 ml. of buffered Bonner's solution (NaCl 0·6 gm.; KCl, 0·75 gm.; $CaCl_2$, 0·3 gm.; distilled water, 1,000 ml., buffered with tris at a final concentration of 40 mM, pH 6·5). The suspension was then centrifuged 2–3 times at about 500 g for 5 minutes in order to separate the bacteria from the myxamoebae. After washing, the cells were resuspended in experimental solution.

The chelating agent used in these experiments was disodium ethylenediamine tetra-acetate (EDTA). EDTA solutions were made up in calcium-free tris-buffered Bonner's solution. The pH of all solutions was routinely checked at the beginning and end of each experiment and found to vary by no more than 0·2 pH units.

Cells and pseudoplasmodia were fixed for microscopic examination by leaving overnight in 0·5 per cent. silver nitrate solution. After alcohol dehydration and mounting in balsam, the material was 'developed' by exposure to a 100-watt incandescent bulb for 18 hours.

Experiments will be described to demonstrate the effects of EDTA in three different situations: (1) as a submersion fluid for the migratory pseudoplasmodium, (2) in the medium of a hanging-film preparation during the aggregative phase, and (3) when included in the agar substratum during aggregation.

RESULTS AND DISCUSSION

If a pseudoplasmodium is transferred without damage from its agar substratum to the bottom of a 5-ml. Beckman cup, and Bonner's solution is carefully poured over it, the slug behaves as if it had a sheath or coating of a non-wettable substance. It can be submerged only with difficulty because of the forces of surface tension which buoy it up. Once submerged, the sausage-shaped pseudoplasmodium immediately begins to round up, and within about an hour it has become quite spherical in shape. Its surface remains smooth and unbroken, and no further change occurs over a period of several hours (Plate, fig. A). Mild agitation produces no effect. If the sphere is cut with a glass needle, healing occurs rapidly, the cut surfaces either rejoining, or smoothing over within one to two hours.

If, instead of Bonner's solution, 5 mM EDTA is used as a submersion fluid, markedly different results are observed. Within minutes the entire surface of the pseudoplasmodium begins to disintegrate, transforming the once smooth, integrated slug into a ragged mass of violently active individual cells (Plate, fig. B). Mild agitation now separates the cell mass into smaller clumps. Even without agitation, the slug soon begins to break up into smaller groups of cells until, after several hours, only a single layer of active cells remains on the bottom of the dish. If a drop of trypan blue is added to the solution and (after a few minutes) these cells are pulled apart with glass needles, slightly stained fibres of gummy material are drawn out between the cells. Whether this is true intercellular matrix, extruded mucus (Auerbach & Grobstein, 1958), or simply remnants of the slime sheath, is not at present clear.

The activity of these cells in EDTA consists largely of the formation and rapid unoriented motion of hyaline lobopodia or surface 'blebs'. This phenomenon is apparently similar to that seen in embryonic amphibian cells disaggregated by high pH (Holtfreter, 1948; Steinberg, 1958), and the present author has observed it in ectoderm cells dissociated from early neurulae of *Amblystoma maculatum* by exposure to 10 mM EDTA. It has also been described after treatment with EDTA of cultured chick fibroblasts (Dornfeld & Owczarzak, 1958) and adult and fetal guinea-pig liver-cells (Peters, 1958). Finally, Moscona & Moscona (1952) have reported identical behaviour of chick limb-bud cells freshly disaggregated with trypsin.

Since EDTA appears to disaggregate the pseudoplasmodium of *D. discoideum* just as it does the tissues of metazoan forms, two questions follow: (1) what is the effect of this agent on the myxamoebae before aggregation, and (2) are the myxamoebae able to aggregate in its presence?

In a hanging-film preparation vegetative cells adhering to the glass have a characteristic form and behaviour. They move very slowly and extend many short and finely pointed or wider and obtusely pointed pseudopodia. There is little evidence of contractile vacuole activity (Plate, fig. C). In a matter of a few hours (the exact time depending on cell density, number of residual bacteria, and other factors) these cells enter the adhesive phase, in which they flatten against the glass, take on a more elongated form, and now have mostly short, wedge-shaped pseudopodia. Contractile vacuoles are present in most cells. The early aggregative cells form contacts with their neighbours and soon begin to aggregate together into clumps and streams. If two such cells draw apart, a strand of material may be seen between them (Plate, fig. D), which appears to form from a 'sticky' surface substance. The alternative possibility that such strands represent true cytoplasmic extensions cannot be dismissed, however, until further evidence is available. Still later, smooth streams of oriented cells form, which flow toward aggregation centres.

If the Bonner's solution covering vegetative cells on the glass is replaced with 5 mM ETDA, the cells pull in pseudopodial extensions and round up. Most of them fall free of the glass, coming to rest at the air–water interface. Within minutes some of the cells begin surface bubbling, throwing out hyaline lobopodia and writhing in the manner already described for disaggregated pseudoplasmodium cells (Plate, fig. E). In a few hours in 5 mM EDTA the remainder of the cells become active, and some cell death occurs. EDTA at a concentration of 2 mM has a similar effect; extensive cell death is, however, somewhat delayed. One millimolar EDTA appears to be a critical concentration, which causes cell surface activity and writhing, but which, none the less, allows the myxamoebae to migrate together into loose heaps of cells, apparently in response to an acrasin gradient. Very little cell death occurs. These abortive centres never develop the smooth surface which is so characteristic of the normal aggregate. However, if such a centre forms in contact with the air–water interface, a minute, poorly formed sorogen is occasionally formed. Whether differentiation of myxamoebae into stalk cells and spores occurs in these structures is not known at present. If, instead of EDTA alone, EDTA plus an equimolar concentration of calcium chloride is used for washing the vegetative cells, no deleterious effects are seen, no changes in cell shape or behaviour occur, and normal aggregation and fruiting ensue. Fig. F of the Plate shows a normal aggregation centre with oriented cells streaming into it. In this hanging-film preparation the liquid phase is calcium-free Bonner's solution. Fig. G of the Plate shows a similar preparation in which the liquid phase contains EDTA and calcium chloride at a concentration of 10 mM each.

A second means of determining the effect of EDTA on aggregation of the myxamoebae, which obviates the difficulty of having most of the cells come off the glass coverslip, is to solidify the EDTA or calcium-free Bonner's solution with 2 per cent. agar, and plate the washed vegetative cells on to this non-nutrient gel in Petri dishes. Fig. H of the Plate shows streams and smooth aggregation centres forming 6 hours after cells were plated on to calcium-free Bonner's solution in agar. These produced normal mature fruiting bodies within the next 18 hours. Cells plated on to 2 mM EDTA-agar form low mounds as centres, but incoming streams are poor and loosely formed (Plate, fig. I). These centres continued developing into poorly formed small sorogens or abortive sorocarps, most of which fell over on to the agar before fruiting was completed. On 5 mM EDTA-agar, no streams are seen contributing to the loose cellular aggregates. The stage pictured in Fig. J of the Plate was reached 6 hours after the washed cells had been plated out. Although these cells remained alive and continued writhing during a period of 28 hours of observation no further development took place. Ten or twenty millimolar EDTA in agar completely prevents aggregation. On the other hand, as noted previously, 10 mM $CaCl_2$ added to 10 mM EDTA in agar completely protects the cells from deleterious effect, aggregation and culmination occurring normally.

The apparent need for a greater effective concentration of EDTA in agar, compared with conditions in the hanging-film preparations, is at first confusing. Cells on a 5 mM EDTA-agar plate are in contact with a reservoir of about 50 micromoles of the substance, whereas cells in a film of solution on the underside of a coverslip are in contact with a total of less than 0·1 micromoles of the chelating agent. However, analysis of the agar used for these plates indicated a high content of heavy metal and alkaline earth cations. Every one of these cations should be expected to complex with one molecule of EDTA, preventing it from removing cellular calcium. This observation apparently also explains why extensive cell death occurred in 5 mM EDTA on glass but not on agar. It is interesting to note here that Ennis & Sussman (1958) have recently pointed out that alkaline earth cations, especially calcium, are required for motility of myxamoebae on agar.

There are at least five means whereby a chelating agent such as EDTA might be expected to disturb normal intercellular adhesion.

(1) Dissolution of mucoid or proteinaceous extracellular ground substance or sheath, if such material depends for its integrity on bound cations.

(2) Chelation of intercellular calcium or magnesium bridges.

(3) Chelation of alkaline earth ions bound in the cell cortex or surface, and maintaining a semi-stable, pseudoparacrystalline pattern of surface charges.

(4) Enzyme inhibition by the removal of metallic coenzymes.

(5) Activation of lytic enzymes by the removal of cationic inhibitors.

The role and nature of the mucus material of the slime mould pseudoplasmodium remain obscure. However, the slime of the snail has been carefully analysed and shown to contain a mucopolysaccharide component linked to an acidic protein by calcium (Kwart & Shashoua, 1957). In fact, a recent reviewer of the literature concerning intercellular material concludes that inorganic cations commonly play a part in the coacervate formation between negative mucopolysaccharides and proteins (Rinaldini, 1958). Thus it is not unlikely that point 1 (above) is involved in the effects noted in the present experiments.

The possibility that cellular adhesion results from binding across intercellular calcium-ion 'bridges' has been suggested by several authors (Coman, 1954; Steinberg, 1958; DeHaan, 1958). There is no evidence from the present experiments to refute this idea. It is not, however, sufficient to account for the observed cell-writhing and surface instability in vegetative cells, that is, in cells *not* having been dissociated from a previous state of aggregation. It would seem more likely that the break-down of cell surface stability and disaggregation both derive independently from the action of the disaggregating agent, or that the surface activity is the cause rather than the effect of disaggregation. Further support for this idea stems from Holtfreter's (1948) observations on amphibian cells treated with alkaline solutions: that part of the cell surface covering a hyaline lobopodial bulge was not adhesive, in contrast to the remainder of the membrane.

Surface instability might be accounted for by points 3, 4, or 5 (see above). Calcium ions bound to monomolecular films of cephalin are known to stabilize such layers at an oil–water interface (Alexander, Teorell, & Aborg, 1939), and to prevent the formation of active myelin bodies by submerged globules of crude lecithin (Holtfreter, 1948). Heilbrunn (1956) has demonstrated the need for alkaline earth cations in the gelation of the cell cortex in amoebae, and Gross (1957) has suggested that competition for binding sites on cytoplasmic colloids between organic cations and calcium might be involved in the mechanism of sol-gel transformation. In so far as these phenomena are related, removal of cations from the cell membrane would not be consistent with the maintenance of stability.

It is now recognized that all ATP-linked enzyme reactions require calcium or magnesium (Ingraham & Green, 1958). Furthermore, ATP-ase activity has been reported to be localized in the cell membranes of mammalian liver and kidney cells (Essner, Novikoff, & Masek, 1958; Spater, Novikoff, & Masek, 1958). If phosphate-bond energy is required for the maintenance of membrane stability and intercellular adhesion, then calcium removal, by inhibiting ATP-dependent reactions in the cell membrane, would produce the observed surface activity and disaggregation. This possibility is made more plausible when it is recalled that in embryonic tissues, before specialized intercellular adhesive structures or interdigitations have formed, treatment with metabolic inhibitors frequently leads to cell dispersion (Spratt, 1950).

That point 5 (see above) may be operative in the activation or unmasking of lytic enzymes by the chelation of cations is suggested by the recent work of Bangham & Dawson (1958). These workers demonstrated that pure lecithin is not attacked by lecithinase as long as the phosphatide molecule maintains a neutral or net positive electrophoretic charge. However, addition of certain negatively charged surface active agents, or the removal of calcium and other cations, producing a net negative charge on the lecithin, permitted its degradation by the enzyme. The presence of lecithin and other phosphatides in the plasma membranes of many forms is now commonly accepted (see DeHaan, 1958, for literature).

It seems reasonable to conclude from the present work that the cells of the slime mould *D. discoideum* react to a powerful chelating agent in a manner apparently analogous to that of metazoan cells. Further investigation of problems of cell movement and adhesion, using the slime moulds as experimental material, may prove fruitful, and the general application of information derived from such studies appears to be justified until other differences in basic mechanism are uncovered.

SUMMARY

1. Vegetative myxamoebae and pseudoplasmodia of the slime mould *Dictyostelium discoideum* were treated with the chelating agent ethylenediamine tetra-acetic acid.

2. The myxamoebae first respond by rounding up, and then by throwing out active, continuously moving, hyaline lobopodia. At high concentrations of EDTA the cells are unable to migrate, although cellular 'writhing' continues for hours. At lower concentrations writhing also occurs, but the cells are able to move together into loose heaps.

3. A migratory pseudoplasmodium immersed in 5 mM EDTA begins disaggregating rapidly. Its smooth surface breaks down and individual cells exhibit

membrane activity similar to that described above. The cells lose their attachments to one another, and are maintained in a loose pile only by a tenuous matrix in which they appear to be embedded. Mild agitation now easily separates them into single cells and small clumps of cells.

4. The effects noted on both myxamoebae and pseudoplasmodia are reversible by replacing the EDTA with Bonner's solution. Moreover, preparations treated with EDTA in the presence of equimolar concentrations of calcium chloride are unaffected.

5. How the removal of cations from cells might result in their dissociation from one another is discussed. It is suggested that EDTA may have its effects by (*a*) dissolving extra-cellular ground substance or sheath material, and (*b*) producing a state of instability in the cell membrane allowing continuous pseudopodial activity and distortion of relations between apposed cell surfaces.

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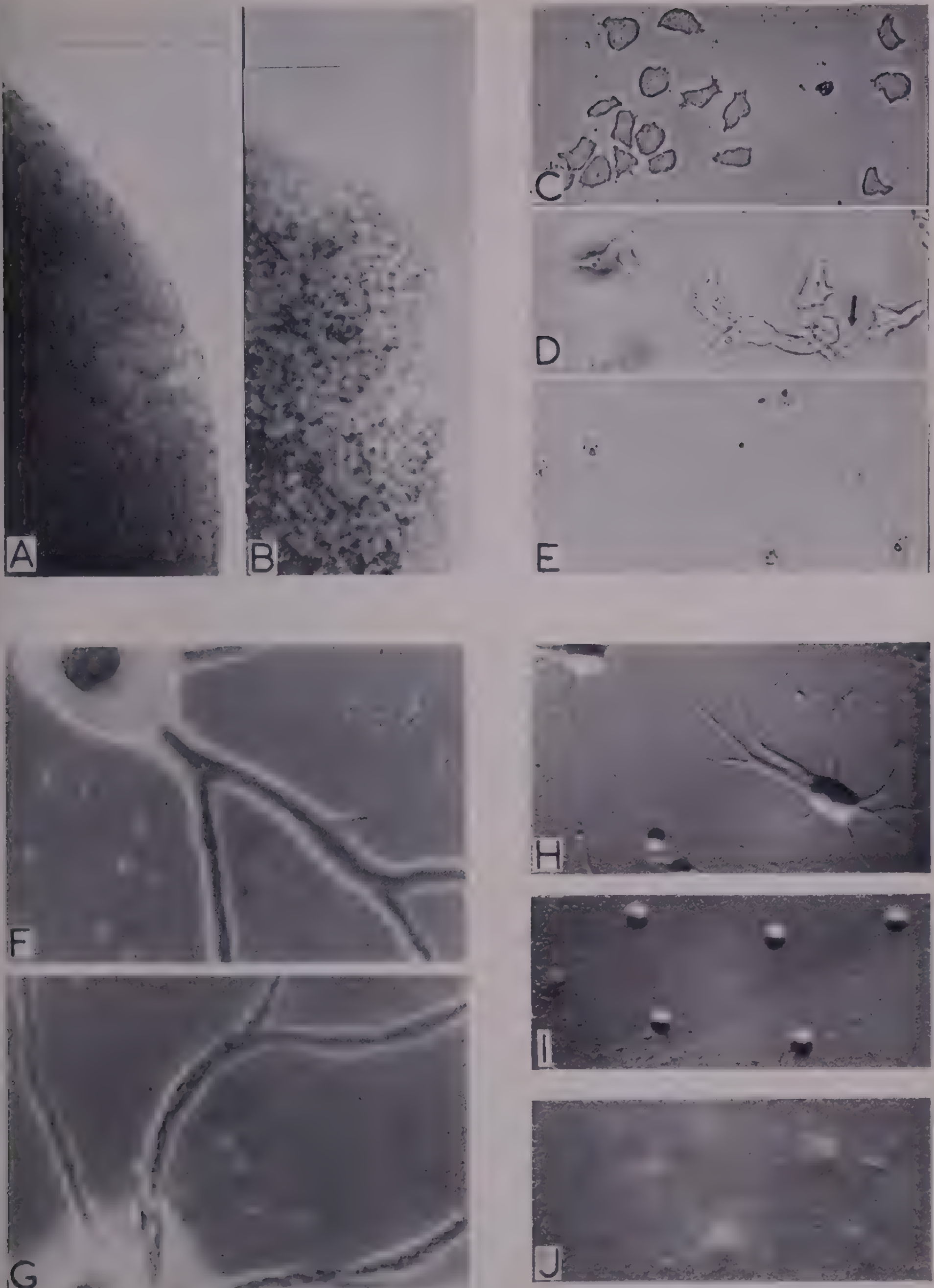

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EXPLANATION OF PLATE

FIG. A. Optical section through the edge of a rounded pseudoplasmodium after 2 hours in tris-Bonner's solution. Fixed in 0·5 per cent. silver nitrate. ×400.

FIG. B. Pseudoplasmodium in initial stage of disaggregation. Fixed 8 minutes after submersion in 5 mM EDTA. ×400.

FIG. C. Living vegetative cells, in a hanging-film preparation. Note many short, pointed extensions. ×400.

FIG. D. Living myxamoebae in early aggregative phase. Note strand of intercellular material at arrow. ×400.

FIG. E. Living vegetative cells in 5 mM EDTA. Note hyaline surface blebs. ×400.

FIG. F. Control aggregation centre with streams. On coverslip, with hanging-film of calcium-free Bonner's solution. ×150.

FIG. G. Aggregation centre with streams. On coverslip with hanging-film of 10 mM EDTA plus 10 mM calcium chloride. Note lack of deleterious effects. ×150.

FIG. H. Control aggregation centre with streams. On non-nutrient agar. ×30.

FIG. I. Aggregation centres with individual cells migrating in. On 2 mM EDTA-agar. ×30.

FIG. J. Loose cell aggregates. On 5 mM EDTA-agar. ×30.

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The Formation of the Second Maturation Spindle in the Eggs of *Succinea*, *Physa*, and *Planorbis*

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WITH TWO PLATES

IN a previous communication (Raven, Escher, Herrebout, & Leussink, 1958) the maturation of the egg was described in *Agriolimax reticulatus*, *Limax flavus*, and *Limnaea stagnalis*, with special regard to the formation of the second maturation spindle. In the three species the second maturation spindle arises by direct transformation from the deep centrosphere of the first maturation amphiaster. In *A. reticulatus* and *Limax flavus* this centrosphere contains a pair of centrioles which move apart to opposite poles of the developing spindle. This is symmetrical and develops an aster at both ends, arising in the cytoplasm outside the original centrosphere.

In *Limnaea stagnalis* the centrosphere of the first maturation amphiaster contains only one centriole, which remains undivided. When the centrosphere elongates, the centriole moves as a whole to its outer pole. The spindle developing from the centrosphere is therefore asymmetrical and egg-shaped, and aster formation occurs at its pointed outer end only. The inner aster of the second maturation spindle is provided by the sperm aster, which fuses secondarily with the blunt inner end of the spindle.

In discussing this aberrant course of the second maturation division in *L. stagnalis*, it was concluded that its primary cause is the failure of the egg cytocentre to divide properly. This causes the lack of astral radiations at the deep end of the developing second maturation spindle, and enables the sperm aster to fuse with this end of the spindle. It was argued that a mutation bringing about a precocious inactivation of the egg cytocentre, in conjunction with the circumstance that a sperm aster, free to take over the task of the failing deep maturation aster, is present at the right moment, might have led to this course of events. As it appeared conceivable that a modification of development occurring at such an early stage might have a profound evolutionary significance, it seemed important to investigate the taxonomic distribution of this peculiar mode of formation of the second maturation spindle.

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Since the completion of our previous paper eggs of various pulmonates, both Basommatophora and Stylommatophora, have therefore been fixed at various moments during the course of the maturation divisions. The study of these eggs in sections soon revealed that the above formulation of the problem is rather over-simplified. We had tacitly assumed that the mode of formation of the second maturation spindle as observed in *Limax* and *Agriolimax* is the 'normal' one in pulmonates, while the relationships found in *Limnaea stagnalis* constitute a deviation of the process occurring in a limited number of species of this subclass. However, to our surprise it appeared that this early and quite fundamental process exhibits a bewildering variation among the species. Its study in as large a number of species as possible seems necessary before any generalizations can be framed. In view of the fundamental importance of all data relative to the processes of meiosis and mitosis, such a study may yield important results.

In this paper the formation of the second maturation spindle in four pulmonates will be described: the stylommatophore *Succinea putris* L., and the basommatophore species *Physa acuta* Drap., *Planorbis planorbis* L., and *P. corneus* L.

MATERIAL AND METHODS

The eggs of *Succinea putris*, *Planorbis planorbis*, and *P. corneus* were obtained from snails caught in the neighbourhood of Utrecht and kept for some time in the laboratory. Those of *Physa acuta* were from specimens living in the aquaria of the Institute.

Since the formation of the second maturation spindle is a process which, especially in some species, takes place very soon after oviposition, it was important to be able to obtain the eggs at will at the desired moment. To that end, methods to stimulate oviposition in these snails have been worked out by Lucy P. M. Timmermans (1959).

The eggs of the land snail *Succinea putris* are laid, preferentially within moist moss, in irregular masses, containing from 20 to 60 egg capsules. These capsules are each surrounded by a layer of jelly (Jura & George, 1958), by which they are cemented together into a loose aggregate. The spherical egg capsules have a diameter of about 1 to 1·3 mm. They have a thick, elastic, transparent wall, which is not encrusted with lime. The egg cells have a diameter of about 130 μ.

The egg-masses of *Physa acuta* are gelatinous, oblong, and from 10 to 18 mm. in length. They may contain from 15 to 50 egg capsules, lying rather freely in the common jelly. The egg capsules are oblong and about 0·7 × 0·9 mm. in size.

The egg-masses of *Planorbis planorbis* are flat and disk-shaped, and have a diameter of from 3 to 5 mm. The egg capsules lie side by side in one layer, close together. There are about 15 to 25 egg capsules, each about 0·5 mm. in diameter. The egg cells have a diameter of 110 30 μ. In the first 15 minutes after oviposition the egg masses are whitish and opaque; then they become transparent, with a yellowish tinge.

P. corneus also has flat, disk-shaped egg-masses, containing one layer of egg capsules (cf. Jura & George, 1958). The egg-masses are about 8 to 10 mm. in diameter, have a reddish tinge, and contain from 15 to 25 egg capsules. The outer jelly layer of the egg-mass is rather rigid. The egg capsules are transparent and 1·4–1·6 mm. in size. The diameter of the egg cells is about 140 μ.

A freshly laid egg-mass was observed under a microscope, and the time of formation of the first polar body in the majority of the eggs was noted. This time was taken as the starting-point of the subsequent observations; all indications of time mentioned below are counted from the moment of extrusion of the first polar body. Batches of eggs were fixed at regular intervals during the course of the second maturation division. Owing to the considerable variations in stage of development among eggs of the same batch, the maturation age of the eggs provides only a rough estimate of their stage of development.

The first polar body is formed in *Succinea putris* about one hour after oviposition; in *Physa acuta* 5–15 minutes, in *Planorbis planorbis* about 15 minutes, and in *P. corneus* 10–20 minutes after oviposition. The second polar body is extruded about 65 minutes after the first in *Succinea putris*; about 35 minutes in *Physa acuta*; 50 minutes in *Planorbis planorbis*, and 55 minutes in *P. corneus* (at an average temperature of 20° C.).

Before fixation the eggs must be decapsulated. In *Succinea putris* and *Physa acuta* this can be easily done in the same way as in *Limnaea*: the egg-mass is rolled out on a glass plate covered with moist filter-paper, so that the egg capsules come to lie apart. In *Physa acuta* care must be taken not to damage the egg capsules, as the capsule membrane is rather delicate in this species; in *Succinea* it is thick and resistant. The egg cells are isolated by pricking the capsule membrane; this is most easily done after the latter has become slightly wrinkled by drying. In *Planorbis planorbis* and *P. corneus*, decapsulation is only possible during the first 15–20 minutes after oviposition, before the outer envelope of the egg-mass has hardened. With watchmaker's forceps this envelope is removed; then the egg capsules are pricked under water.

RESULTS

Succinea putris

In the eggs of the first batch, fixed at 15 minutes, the first polar body has just been constricted off. It is still connected with the egg surface by a stalk or a 'mid-body'. The deep aster of the first maturation spindle lies beneath the surface at the animal pole, often slightly to one side of the attachment of the polar body and the spindle remnant. This seems to be a peculiarity of the *Succinea* egg, as it has never been observed in other species. The aster is provided with a dense centrosphere of moderate size. This is slightly flattened and elongated parallel to the surface. Two very minute, hardly visible centrioles are found near the ends of its longer axis. The dyads closely apply themselves against its outer surface in a loose group; they even seem partly to have invaded the area of the centrosphere (Plate 1, fig. A). The aster and spindle remnant are surrounded by a dense area of the cytoplasm; many mitochondria have accumulated in this region.

In the next few minutes the spindle remnant disappears. The centrosphere with the dyads approaches the surface and comes to lie immediately beneath the egg cortex, flattening still more against it. The astral radiations of the old maturation aster become blurred and indistinct, but in some cases a new orientation of astral fibres is indicated near the ends of the elongated centrosphere where the centrioles are barely visible as ill-defined and somewhat darker dots.

Apparently this does not yet give rise to the asters of the second maturation spindle, however, for in subsequent batches all astral radiations have disappeared. As a matter of fact, in the eggs fixed at 30 minutes, the dyads are found in an area of clear protoplasm, lying immediately beneath the surface near the animal pole but often somewhat eccentric with respect to the first polar body (Plate 1, fig. B). This clear area, which may be rather irregular in outline, possibly represents the shrunken centrosphere. Subsequently it once more shifts its position and sinks somewhat below the surface but remains in the region of dense animal cytoplasm (Plate 1, fig. C). Here two small asters appear at opposite ends of the clear area, and become connected by a spindle. Part of the spindle fibres traverse the clear area and connect with the dyads, while other fibres run along its outer border (Plate 1, fig. D). The spindle, which may at first have a tangential or oblique position, then rises to the surface and places itself at right angles to it (Plate 1, fig. E). The dyads arrange themselves in the equatorial plane of the spindle, while the latter increases considerably in size. The aster at the outer end of the spindle flattens against the surface. The inner aster grows considerably in size and develops a large clear centrosphere, in the middle of which the centriole is now distinctly visible (Plate 1, fig. F).

During the early stages of the formation of the second maturation spindle no sperm aster is present in the egg. It appears for the first time when the second maturation spindle is in metaphase. It is situated at an arbitrary place in the cytoplasm, occasionally quite near to the second maturation spindle (Plate 1, fig. F), but mostly at a greater distance. During anaphase of the second maturation division it grows somewhat in size and develops a distinct centrosphere, which may often show a tendency to reduplicate. Immediately after the extrusion of the second polar body this centrosphere becomes strongly vacuolized, and the whole sperm aster rapidly disappears without leaving any traces.

Physa acuta

Early development in *Physa acuta* is much more rapid than in other species. The second polar body is extruded 35 minutes after the first. The formation of the second maturation spindle takes place in the first 20 minutes after the extrusion of the first polar body.

At telophase of the first maturation division the dyads reach the outer margin of the deep centrosphere. This is small, rather dense, and ellipsoid in shape (Plate 1, fig. G). No distinct centrioles are visible in it.

Very soon, this centrosphere transforms into the second maturation spindle. It elongates in a direction parallel to the surface and becomes spindle-shaped. At the same time a longitudinal striation appears in it. Figure H of Plate 1 shows an early stage of this transformation. The developing second maturation spindle, and the dyads situated against its outer side, are still connected with the egg surface by a remnant of the first maturation spindle. Where this reaches the surface there is a dark 'mid-body', with which the first polar body is connected (not in this section).

Figure J of Plate 1 shows a slightly further advanced stage. The developing second maturation spindle has become somewhat longer, and the spindle fibres are more distinct. At both its ends a small dark granule, presumably a centriole, has now become visible, and small asters are forming around them. But the spindle still lies in a superficial position parallel to the surface, and the dyads are situated against its outer side.

In the following few minutes the spindle begins to rotate and places itself at right angles to the surface. At the same time the dyads invade the spindle area and arrange themselves more or less irregularly in the middle region of the spindle. The asters grow in size and the outer aster becomes attached to the egg surface, where a small indentation may temporarily be formed (Plate 1, fig. K). The dyads arrange themselves into a metaphase plate; sometimes this may occur already when the spindle has not yet completed its rotation (Plate 1, fig. L), but in other cases it only takes place after the spindle has become attached to the surface (Plate 1, fig. K).

A sperm aster does not appear in the egg of *P. acuta* before the anaphase of the second maturation division. At first it is a very small structure, but soon it grows in size and develops a centrosphere. Immediately after the extrusion of the second polar body, however, a vacuolization occurs in its centre, and the sperm aster disappears. As early as 10 minutes after the formation of the second polar body no trace of the sperm aster remains.

Planorbis planorbis

The egg of *Planorbis planorbis* is very suitable for the study of its maturation divisions. It gives images of great clearness and beauty. This, together with its peculiar and quite unconventional mode of formation of the second maturation spindle, makes it an excellent object for studies relating to maturation, fertilization, and cell-division.

In the eggs of the first batch, fixed 15 minutes after the extrusion of the first polar body, the spindle remnant of the first maturation division has already disappeared. The deep aster that remained in the egg has risen to the surface and flattened itself against it. It has a large, round, disk-shaped, and rather dense centrosphere; the boundary between centrosphere and astral rays is not very sharp. In the middle of the centrosphere lies a somewhat darker but rather vaguely delineated body, apparently a centriole. It is always single. The dyads are arranged in a more or less regular circle around the centrosphere, at the boundary between the latter and the astral rays (Plate 1, fig. M).

In addition to the maturation aster, a second large aster is present in each egg. It has a variable location, often near the centre of the egg, but sometimes at a short distance beneath the maturation aster. The fact that it is connected with the sperm tail in most cases (Plate 1, fig. P; Plate 2, fig. A) proves that it is the sperm aster. It is more or less reduplicated in all cases. Sometimes it has an elongate or biscuit-shaped centrosphere in which two rather large, but ill-defined, centrioles can often be distinguished (Plate 1, fig. N). In the middle, between the two centrioles, a longitudinal striation may appear, forming a short central spindle (Plate 1, fig. P). Finally, the centrioles may move farther apart, the central spindle elongates, and the aster divides, in this way forming a beautiful sperm amphiaster (Plate 2, fig. A). It must be emphasized that this is an achromatic spindle, as the sperm nucleus is still lying as a compact dark body somewhere beneath the egg cortex and remains so until after the extrusion of the second polar body, as it does in all pulmonates.

The mitochondria of the egg are accumulated around both the maturation aster and the sperm aster, often penetrating in rows between the astral rays.

Starting from this situation, which prevails shortly after the extrusion of the first polar body, the following processes lead to the formation of the second maturation spindle. The dyads begin to move centrifugally along the astral rays of the maturation aster, thereby widening their circle (Plate 1, figs. O, P).

At the same time the sperm amphiaster rises to the animal pole and comes to lie close beneath the maturation aster. Now delicate fibres are 'spun' between the centre of the maturation aster and the sperm amphiaster, spanning the intervening stretch of cytoplasm. When the sperm amphiaster lies more or less horizontally and its two poles are at about the same distance from the centre of the maturation aster, these spindle fibres may at first run divergently from the latter to both sperm centrioles (Plate 1, fig. P). Later, two different possibilities may be distinguished. When the sperm aster is more or less horizontal and its centrioles lie near together, the fibres of the maturation spindle seem to end indiscriminately along the whole middle region of the sperm amphiaster (Plate 2, fig. B). When the poles of the sperm amphiaster are farther apart, however, and especially when it is situated obliquely or vertically, the maturation spindle is formed between the centre of the old maturation aster and the nearest pole of the sperm amphiaster (Plate 2, figs. A, C). So we get the uncommon situation of a spindle one of the poles of which is itself a (more or less well-developed) spindle.

The maturation spindle formed in this way is at first achromatic, but soon it becomes 'colonized' by the dyads. In their outward migration along the rays of the maturation aster the dyads move at first immediately beneath the egg cortex, against which the aster remains closely applied (Plate 2, fig. A). But then the ends of the astral rays bend inwards, taking along the dyads (Plate 2, fig. B), and the latter reach the outer surface of the developing second maturation spindle, which has in the meantime increased in girth and has become more distinctly visible. In some eggs several of the dyads have reached the spindle (Plate 2, fig. C), whereas other dyads still lie in the peripheral parts of the maturation aster. Finally, all dyads have reached the outer surface of the maturation spindle, and begin to invade the spindle area. Then they arrange themselves into a metaphase plate.

Figure D of Plate 2 shows the second maturation spindle in early anaphase. Its inner pole is formed by the sperm amphiaster, which has two distinct centres connected by a central spindle. The latter at this stage begins to disintegrate, its spindle fibres becoming more or less blurred and apparently breaking up into smaller fragments. The maturation spindle is mainly centred towards the left pole of the sperm amphiaster. The outer aster of the maturation spindle has developed from the original deep aster of the first maturation spindle. As a matter of fact, during the later phases of the migration of the dyads the astral radiations have become somewhat less distinct, but at no stage have they disappeared altogether, and now they begin to become more pronounced again.

A late anaphase stage is shown in Plate 2, fig. E. The sperm amphiaster has two large clear centrospheres, each containing a dark rather ill-defined centriole, and lying obliquely one above the other. They are separated by the remnants of the central spindle, which run as a dark granular band between them. The maturation spindle is connected with the upper half of the sperm amphiaster.

In the next few minutes, while the second polar body is being formed, the two centrospheres of the sperm amphiaster unite to a single spherical clear area with a finely reticular structure, in which a band of coarse granules forms the last remnant of the former central spindle. On either side of this band a centriole is still seen; they have even become much more clearly defined than at previous stages, and present themselves as compact, rather large, and globular bodies. Both sperm centrioles are visible in Plate 2, fig. F, the second polar body having just been pinched off.

Immediately after the extrusion of the second polar body the egg chromosomes begin to swell into karyomeres. At the same time the sperm nucleus also swells. Temporarily it has the appearance of a closely packed group of karyomeres, forming a morula-like body; with further swelling these karyomeres unite to a polymorphic male pronucleus. This stage has been reached 20 minutes after the extrusion of the second polar body. The egg karyomeres are still separate vesicles at this time, but 10 minutes later they too have fused to a polymorphic pronucleus.

After the extrusion of the second polar body the deep aster of the maturation spindle (the former sperm amphiaster) moves somewhat below the surface. Its astral radiations soon begin to disappear. Its centrosphere forms a large, clear, spherical area, finely reticulate in structure, but interspersed with coarse granules. Two centrioles may still be seen in it as distinct dark globules (Plate 2, fig. G). Simultaneously with the swelling of the sperm nucleus, the latter begins to migrate from its subcortical position towards the centre of the egg, where it comes to lie in the centrosphere of the sperm aster. This stage has been reached 20 minutes after the extrusion of the second polar body. Then the centrosphere with the male pronucleus migrates towards the animal pole, where it forms a clear crescent-shaped zone surrounding the two pronuclei. Twenty minutes later this clear zone has disappeared and is replaced by the dense animal pole plasm which has now accumulated at the animal pole.

Planorbis corneus

Although the formation of the second maturation spindle in *Planorbis corneus* occurs essentially in the same way as in *P. planorbis*, the former species is much less favourable material owing to different staining properties.

In all eggs fixed 10 minutes after the extrusion of the first polar body, the deep aster of the first maturation spindle lies closely beneath the surface at the animal pole. It is of moderate size and has, as a rule, no clear centrosphere, the astral rays continuing towards the centre of the aster. The dyads are situated in a narrow circle around this centre (Plate 2, fig. H).

In most eggs a small sperm aster is found. It has no distinct centrosphere. It has a variable location; sometimes it lies at a short distance beneath the maturation aster (Plate 2, fig. H), but as a rule it is situated in other parts of the egg and shows no topographic relations to the maturation aster. In most cases the sperm aster is a single structure; only in a few instances does it show a beginning reduplication of its centre.

In the next 10 minutes the dyads begin to move centrifugally along the rays of the maturation aster, arranging themselves in a wide circle in its periphery (Plate 2, fig. J). At the same time the sperm aster slowly approaches the maturation aster and comes to lie beneath it. Simultaneously, it grows in size, and a clear centrosphere appears in its centre (Plate 2, fig. J). The number of sperm asters showing reduplication increases; in extreme cases this may lead already at this stage to the formation of a small sperm amphiaster, consisting of two asters with distinct centrospheres and connected by a short achromatic central spindle.

When the sperm aster has approached the maturation aster to within a small distance, delicate spindle fibres begin to be 'spun' between the two. If the sperm aster is double, they are directed to one of its centres. At the same time the astral rays of the maturation aster bend inwards and the dyads, moving along them, reach the outer surface of the developing second maturation spindle. In the egg of Plate 2, fig. K, this has occurred with some of the dyads, but the majority of them still lie in the peripheral parts of the maturation aster.

In this way the second maturation spindle becomes 'colonized' by the dyads. This stage has been reached at about 25 minutes. The dyads, first lying at the surface of the spindle, then gradually penetrate into its substance and arrange themselves more or less irregularly along the spindle fibres (Plate 2, fig. L). At 40 minutes they begin to concentrate in the equatorial region (Plate 2, fig. M), and so the metaphase stage is reached. The outer aster of the spindle is the former deep aster of the first maturation spindle; its inner aster is formed by the sperm aster, which is now more or less reduplicated in all cases, ranging from a slight dumb-bell shape of its centrosphere to a well-developed sperm amphiaster with central spindle.

DISCUSSION

It has already been remarked in the introduction to this paper that the present observations have brought to light a quite unexpected variability in the course of the maturation divisions within the group of the pulmonates. They show that some conclusions drawn from our previous investigations were premature, and necessitate a renewed consideration of some points discussed previously.

In a previous paper (Raven, Escher, Herrebout, & Leussink, 1958) it was concluded that in the pulmonates generally the second maturation spindle arises by a direct transformation from the centrosphere of the deep aster of the first maturation spindle. This was shown to be the case in *Limax flavus*, *Agriolimax reticulatus*, and *Limnaea stagnalis*. A similar mode of formation of the second maturation spindle seemed highly probable for other pulmonates from the data found in the literature. Especially the descriptions and illustrations given by Byrnes (1900) for *Limax* (*Agriolimax*) *agrestis* and by Lams (1910) for *Arion empiricorum* leave little doubt that the process in these species occurs in essentially the same way as in *Limax flavus* and *Agriolimax reticulatus* (cf. figs. 10, 15, & 16 of Byrnes, and figs. 42, 48, 56, & 57 of Lams with the photographs in the above-mentioned paper by Raven *et al.*).

The present observations show, however, that we have been overhasty in extending this generalization to the pulmonates in general. It is true that the formation of the second maturation spindle in *Physa acuta*, though differing in some respects, belongs essentially to the same category as that of *Limax*, *Agriolimax*, and *Arion*. In *Succinea putris* this is much less obvious; only with some reservation can we adduce it to the same scheme. But as regards *Planorbis* there is no indication at all of a material continuity between the centrosphere of the first maturation aster and the developing second maturation spindle. Here, therefore, our generalization breaks down altogether; we have to reconcile ourselves with the thought that the second maturation spindle among the pulmonates may apparently be formed in at least two fundamentally different ways.

In *Limax*, *Agriolimax*, and *Arion*, the deep centrosphere of the first maturation spindle at a certain stage contains two centrioles. Some time after the extrusion of the first polar body these centrioles begin to move apart within the area of the centrosphere. At the same time the latter elongates and becomes ellipsoid, with its long axis coinciding with the line connecting the centrioles. Some connecting fibres between the centrioles may form the beginning of a central spindle. The main mass of the spindle arises by the appearance of a longitudinal striation in the substance of the centrosphere, occurring when the centrioles have reached the ends of its long axis.

The formation of the second maturation spindle in *Physa acuta* apparently occurs along the same lines. However, the process does not take place here with such diagrammatic clearness as in the above species. The centrosphere is small and dense, and rather ill-defined with respect to the surrounding aster and cytoplasm. The centrioles do not become visible until they have reached the poles of the developing spindle. Moreover, the whole process occurs very rapidly, the transformation of the centrosphere into the spindle already beginning when it is still connected with the surface by the remnant of the first maturation spindle. But on the whole there is no reason to doubt that the formation of the second maturation spindle in *P. acuta* essentially agrees with that in the slugs.

From the work of Kostanecki & Wierzejski (1896) we may draw the same conclusion with regard to *P. fontinalis*. If one compares figs. 7–12 of their paper with Plate 1, figs. G–L of the present paper, it is apparent that the formation of the second maturation spindle in the two species of *Physa* shows a great resemblance, though some structures, e.g. the centrioles, may appear with greater clearness in *P. fontinalis*.

In *Succinea putris* there are greater deviations from this general scheme. As a matter of fact, it does seem at first that the process will follow the same course. There is a distinct, elongate centrosphere, with minute centrioles near the ends of its longer axis; small asters may even begin to form around these centrioles. But this centrosphere rises to the surface and flattens itself against it. All astral radiations vanish. There remains an area of clear, presumably strongly vacuolated, cytoplasm, in which the dyads are situated. This may either represent the former centrosphere, or it is a new formation due to the accumulation of fluid around the dyads; in the latter case we must conclude that the centrosphere has disappeared altogether. After some time this clear area with the dyads sinks into the depth and gives rise to the spindle, two asters again appearing at opposite ends.

It apparently depends on our interpretation of the 'clear area' just mentioned, whether or not we can compare the formation of the second maturation spindle in *Succinea* with that in the slugs and in *Physa*. If the clear area represents the centrosphere, then there is a fundamental agreement. The first and last phases of the transformation of the centrosphere into the spindle take place more or less according to schedule; but the process is interrupted by a kind of 'rest period' immediately beneath the egg cortex, during which the asters temporarily disappear, and the centrosphere transforms by vacuolization into the 'clear area'.

If, on the other hand, we assume that the centrosphere as such disappears and the clear area arises in another way, it follows that the formation of the second maturation spindle in *Succinea* is essentially different from that in the above-mentioned species. It is not possible at this moment to arrive at a decision between the two alternatives.

In a previous paper (Raven, Escher, Herrebout, & Leussink, 1958) we showed that the formation of the second maturation spindle in *Limnaea stagnalis* differs considerably from that found in slugs. The deep centrosphere of the first maturation spindle contains a single centriole. It lies at first near the centre of the centrosphere, but then it shifts towards the superficial pole of the elongating centrosphere. The formation of spindle fibres begins at this pole and extends only gradually towards the other end of the centrosphere. The spindle formed in this way is asymmetrical, its inner end being blunt and lacking an aster. The sperm aster fuses secondarily with this end and becomes the inner aster of the second maturation spindle.

The formation of the second maturation spindle in *Planorbis planorbis* and *P. corneus* has some points in common with that in *Limnaea stagnalis*, but differs essentially in other respects. As in *Limnaea*, the centriole of the deep aster of the first maturation spindle remains undivided. Moreover, both in *Limnaea* and in *Planorbis* the deep aster of the second maturation spindle is provided by the sperm aster. But there is a great difference in the formation of the spindle proper. While in *Limnaea stagnalis* this arises by a direct transformation from the substance of the centrosphere, there are no indications at all that the centrosphere has anything to do with spindle formation in *Planorbis*. The centrosphere here is disk-shaped, flattened against the surface, and remains in this position while the spindle is being formed. This occurs apparently by the 'spinning' of delicate protoplasmic fibres between the centriole of the maturation aster and one or both centrioles of the sperm aster. No indications have been found that the process begins at either one or both poles; it rather looks like an orientation of the protoplasmic micellae along the 'lines of force' in the 'field' between the poles, occurring simultaneously along their whole length.

In those cases where the two centrioles within the deep centrosphere of the first maturation spindle move to opposite poles of the developing second maturation spindle, new asters are formed at both ends in the cytoplasm outside the transforming centrosphere. This holds for *Limax*, *Agriolimax*, and *Arion*, and apparently also for *Succinea putris* and *Physa*.

In *Limnaea stagnalis* the aster at the outer pole of the developing second maturation spindle is formed in the same way around the centriole lying at this pole. The inner aster is supplied by the sperm aster which fuses secondarily with the spindle.

In *Planorbis* the inner aster of the second maturation spindle is also derived from the sperm aster. Either the whole sperm aster or one of the halves of the sperm amphiaster functions as the deep maturation aster. At the outer end of the spindle a large aster is found; this is no other than the original deep aster of the first maturation spindle, which is taken over as such by the second spindle. As a matter of fact, the astral radiations may temporarily become somewhat vague, so that it is possible that a kind of rejuvenation of the aster occurs; but it is out of the question that the aster disappears entirely and is replaced by a new one in between the two maturation divisions.

Apparently, therefore, variable as is the formation of the second maturation spindle in the pulmonates, the origin of its asters is equally so.

In a previous paper (Raven, Escher, Herrebout, & Leussink, 1958) we have summarized the variations in the development of the sperm aster in various pulmonates. The present observations give new evidence of this variability.

In *Physa acuta* the sperm aster appears during anaphase of the second maturation division. It remains always undivided, and disappears shortly after the extrusion of the second polar body.

In *Succinea putris* the sperm aster becomes visible when the second maturation spindle is in metaphase. It may show a tendency to reduplicate, but again it disappears immediately after the extrusion of the second polar body.

In *Planorbis corneus* a small sperm aster without a centrosphere is already present a short time after the extrusion of the first polar body. It grows rapidly in size and develops a centrosphere; at the same time it becomes more or less reduplicated, ranging from a slightly dumb-bell shaped centrosphere to a well-developed sperm amphiaster. In the meantime it has approached the maturation aster and participated in the formation of the second maturation spindle, whose deep aster it becomes.

Finally, in *P. planorbis* the sperm aster apparently is formed already before the extrusion of the first polar body. Fifteen minutes after first polar body formation it possesses a large centrosphere and is reduplicated in all cases; sometimes it has even at this moment developed into a sperm amphiaster with central spindle. It then approaches the maturation aster, initiates the formation of the second maturation spindle, and supplies its deep aster. Its two centrospheres, each containing a distinct centriole, fuse into a spherical clear area during the extrusion of the second polar body. This area migrates to the animal pole, temporarily forms a crescent-shaped clear zone around the pronuclei, and is then replaced by the developing animal pole plasm.

It is evident that the four species, in the order mentioned, form a series of increasing importance of the sperm aster. In *P. planorbis* it not only appears very early, but reaches a stage of development unprecedented in any pulmonate studied so far. Only the evolution of the sperm aster in *Physa fontinalis*, as described by Kostanecki & Wierzejski (1896), shows some resemblance to that in *Planorbis*. According to these authors, the sperm aster in *Physa fontinalis* divides and forms an amphiaster with central spindle. This may occur at various times, either before or after the extrusion of the second polar body, even as late as the stage at which the male pronucleus approaches the female pronucleus at the animal pole. Anyhow, the sperm amphiaster in all cases gives rise to the first cleavage spindle.

In a previous paper (Raven, Escher, Herrebout, & Leussink, 1958) and in my book (Raven, 1958) I have expressed some doubt on the accuracy of Kostanecki & Wierzejski's observations. However, in view of the great variability which prevails in the course of the maturation divisions in pulmonates, there seems at present to be no reason to think that these observations are incorrect. A re-investigation of the maturation divisions in *P. fontinalis* appears desirable.

Anyhow, the process as it occurs in *Planorbis planorbis* provides a strong argument in favour of the view that, in pulmonates, the poles of the cleavage spindle are also derived by division from the sperm cytocentre. As a matter of fact, the egg cytocentre in this species remains undivided, forms the outer pole of the second maturation spindle, and becomes presumably extruded with the second polar body. In the sperm amphiaster two distinct centrioles are visible. When the centrospheres of the sperm amphiaster have fused to a single spherical clear area after the extrusion of the second polar body, the two centrioles are still clearly visible in it. Presumably they shift together with the clear area and the sperm nucleus towards the animal pole, where the copulation of the two pronuclei occurs. However, at this stage it is impossible to distinguish the two centrioles from other basophil granules in this area. For about 45 minutes, therefore, the centrioles are invisible; then two small asters appear near the pronuclei and give rise to the cleavage spindle. One can hardly doubt that the poles of this spindle are formed by the sperm centrioles. This is the more remarkable as these two centrioles have had a different history, as a rule: for one of the two has formed for some time the deep pole of the second maturation spindle. In view of this circumstance it is conceivable that one of the two sperm centrioles disintegrates, while the other divides once more and forms the poles of the cleavage spindle. Up till now no indications of such a process have been found, however.

A few words remain to be said about the behaviour of the dyads. In *Agriolimax reticulatus* and *Limax flavus* the dyads, after having reached the margin of the deep centrosphere at telophase of the first maturation division, remain as a tightly packed group against the outer surface of the developing second maturation spindle, as a rule near its equatorial region. They do not penetrate into the spindle area until the spindle has been fully formed and has begun its rotation.

The behaviour of the dyads in *Physa acuta* is quite similar to this. In *Succinea putris*, however, it appears that the dyads begin to penetrate into the centrosphere at an early stage. When the latter rises to the surface they come to lie entirely in the area of clear protoplasm which possibly represents the centrosphere. Later, when the second maturation spindle is formed, the spindle fibres penetrate secondarily into this area and connect with the dyads.

In *Limnaea stagnalis* the dyads, at first forming a compact group against the outer side of the centrosphere, become arranged into a more or less regular ring. When the centrosphere begins to elongate, the dyads form a crown around its outer pole. This moves along the outer surface of the developing spindle until it has reached its equatorial region. Only after the sperm aster has fused with the deep end of the spindle do the dyads begin to invade the spindle area.

Finally, in *Planorbis planorbis* and *P. corneus* the dyads first form a narrow ring around the centre of the centrosphere. Then they begin to move centrifugally along the astral rays, in this way widening their circle. At first they move immediately beneath the surface, but then they are taken along by the astral rays bending inwards towards the spindle which has formed in the meantime. So they reach the outer surface of the spindle, after which they penetrate into it.

It is evident, therefore, that the course of the second maturation division in the pulmonates shows a great deal of variation. Various components of the process may vary more or less independently provided, of course, that they fit together in such a way as to ensure the normal completion of the maturation division. Every species shows a species-specific pattern, although smaller variations may occur within each species. This species-specificity is such that it would be possible to construct a key for the identification of the species here described merely on the basis of their second maturation divisions.

This is the more remarkable as we are here dealing with a process taking place at such an early stage of development. In general we are accustomed to the fact that developmental processes in related species show a greater similarity the earlier they take place, in accordance with v. Baer's principle. I know of no other example where such fundamental differences between developmental processes occur at such an early stage.

Of course it might be argued that the development of the new individual does not begin before the formation of the zygote nucleus, so that the maturation of the egg does not represent an early, but rather a very late, stage of development. But such a formal point of view does not discount the fact that differences in developmental processes during egg maturation might profoundly affect the course of subsequent development, e.g. by influencing the localization or the synthesis of cytoplasmic substances. In this way they might be of decisive importance for the determination of species-specific differences. Perhaps such a view might be substantiated by cytochemical investigations during maturation in pulmonates.

SUMMARY

1. Egg maturation has been studied in *Succinea putris*, *Physa acuta*, *Planorbis planorbis*, and *P. corneus*, with special regard to the formation of the second maturation spindle.

2. In *Physa acuta* the second maturation spindle arises by direct transformation from the deep centrosphere of the first maturation amphiaster. This process begins already when the centrosphere is still connected with the surface by the remnant of the first maturation spindle.

3. In *Succinea putris* the deep centrosphere of the first maturation spindle

rises to the surface and flattens itself against it. The dyads come to lie in an area of clear cytoplasm, which subsequently sinks into the depth and gives rise to the spindle.

4. In *Planorbis planorbis* and *P. corneus* the centriole of the deep aster of the first maturation spindle remains undivided. This aster flattens against the surface and becomes the outer aster of the second maturation spindle. Its inner aster is provided by the sperm aster, while the second maturation spindle itself is formed by the 'spinning' of protoplasmic fibres between the centriole of the maturation aster and one or both centrioles of the sperm aster.

5. The sperm aster in *Physa acuta* is a rudimentary structure which appears late, disappears soon, and always remains undivided. In *Succinea putris* the sperm aster appears slightly earlier and may show a tendency to reduplicate. In *Planorbis corneus* the sperm aster appears when the first polar body is extruded. It grows rapidly in size, becomes more or less reduplicated, and participates in the formation of the second maturation spindle. In *P. planorbis* the sperm aster is formed before the extrusion of the first polar body. It reduplicates and forms a beautiful sperm amphiaster with central spindle. It participates in the formation of the second maturation spindle and becomes its deep aster. During the extrusion of the second polar body its two centrospheres, containing distinct centrioles, fuse to a spherical clear area. The sperm nucleus comes to lie in this area, then migrates together with it towards the animal pole. The sperm centrioles presumably form the poles of the first cleavage spindle.

6. In *Physa acuta* the dyads lie at first as a compact group on one side against the developing second maturation spindle, and penetrate only secondarily into the spindle when this is nearly completed. In *Succinea putris* the dyads presumably penetrate into the centrosphere at an early stage. In *Planorbis planorbis* and *P. corneus* the dyads first form a narrow ring around the centre of the maturation aster, then move centrifugally along its astral rays, and are conveyed by the latter bending inwards towards the outer surface of the second maturation spindle.

7. It is concluded that fundamental differences in the course of the second maturation division occur in the pulmonates.

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EXPLANATION OF PLATES

PLATE 1

FIGS. A–F. Formation of second maturation spindle in *Succinea putris*. ×910.

FIG. A. After 15 minutes. Telophase of first maturation division. Dyads against outer side of centrosphere.

FIG. B. After 20 minutes. Astral radiations have disappeared. Dyads in clear area beneath the surface at animal pole.

FIG. C. After 30 minutes. Clear area with dyads has shifted to deeper position; surrounded by dense animal cytoplasm.

FIG. D. After 40 minutes. Transformation of clear area into second maturation spindle.

FIG. E. After 55 minutes. Second maturation spindle has risen to the surface. Dyads still in irregular position in middle region of spindle.

FIG. F. After 80 minutes. Second maturation spindle in metaphase. Deep aster with clear centrosphere and distinct centriole. Sperm aster just beneath deep aster of maturation spindle.

FIGS. G–L. Formation of second maturation spindle in *Physa acuta*. ×910.

FIG. G. After 15 minutes. Telophase of first maturation division. Dyads against outer surface of centrosphere.

FIG. H. After 20 minutes. Centrosphere elongated parallel to surface. Dyads against its outer margin and still connected with 'mid-body' at surface by spindle remnant.

FIG. J. After 20 minutes. Centrosphere transformed into second maturation spindle. Centriole with small aster at either end. Dyads against outer side of spindle.

FIG. K. After 20 minutes. Second maturation spindle has placed itself perpendicular to surface. Dyads have penetrated into spindle, but are still irregularly situated. Indentation of egg surface at attachment of spindle.

FIG. L. After 20 minutes. Rotation of second maturation spindle not yet completed. Dyads arrange themselves in equatorial plane.

FIGS. M–P. Formation of second maturation spindle in *Planorbis planorbis*. ×910.

FIG. M. After 15 minutes. Tangential section. Maturation aster with dyads arranged in circle in circumference of centrosphere. Single centriole in middle of centrosphere.

FIG. N. After 25 minutes. Sperm aster with biscuit-shaped centrosphere containing two centrioles.

FIG. O. After 25 minutes. More or less tangential section. Beginning centrifugal movement of dyads along rays of maturation aster.

FIG. P. After 15 minutes. Maturation aster with single centriole and widening circle of dyads (above). Sperm amphiaster with two centrioles and short central spindle (middle) in connexion with sperm tail (bottom left). Beginning of spindle fibres, running divergently from centre of maturation aster to both sperm centrioles.

PLATE 2

FIGS. A–G. Formation of second maturation spindle and completion of second maturation division in *Planorbis planorbis*. ×910.

FIG. A. After 15 minutes. Maturation aster with dyads along peripheral part of astral rays, closely applied against egg cortex (above). Sperm amphiaster with well-developed central spindle (middle). The left half of the sperm amphiaster is connected with the maturation aster by the spindle fibres of the developing second maturation spindle, the right half with the sperm tail (bottom right, out of focus).

FIG. B. After 25 minutes. Maturation aster with single centriole (above). connected by second maturation spindle with sperm amphiaster possessing two centrioles (middle). Rays of maturation aster bending inwards, taking along dyads.

FIG. C. After 25 minutes. Second maturation spindle, connected with left half of reduplicated sperm aster containing two centrioles. Dyads have reached outer surface of maturation spindle.

FIG. D. After 20 minutes. Early anaphase of second maturation division. Sperm amphiaster with central spindle, showing beginning disintegration of spindle fibres. Deep end of second maturation spindle centred towards left pole of sperm amphiaster.

FIG. E. After 60 minutes. Late anaphase of second maturation division. Sperm amphiaster with two centrospheres, each containing a centriole, and separated by remnants of central spindle. Maturation spindle connected with upper half of sperm amphiaster.

FIG. F. After 60 minutes. Telophase of second maturation division. Centrospheres of sperm amphiaster fused to spherical clear area, containing remnants of central spindle. One sperm centriole just beneath telophase chromosomes, the other one in middle of lower half of clear area.

FIG. G. After 60 minutes. Remnant of sperm amphiaster as spherical clear area, still containing the two sperm centrioles (globular bodies in upper half of clear area).

FIGS. H–M. Formation of second maturation spindle in *Planorbis corneus*. ×910.

FIG. H. After 10 minutes. Maturation aster with dyads near centre of aster (above). Small sperm aster without centrosphere (middle).

FIG. J. After 25 minutes. Maturation aster with dyads along peripheral part of astral rays (above). Sperm aster with centrosphere (middle).

FIG. K. After 15 minutes. Formation of second maturation spindle between maturation aster (above) and sperm aster (below). Some dyads have reached outer surface of maturation spindle (at left), others still in peripheral part of maturation aster (upper right).

FIG. L. After 25 minutes. Dyads have penetrated into maturation spindle. Partial reduplication of sperm aster.

FIG. M. After 40 minutes. Dyads begin to concentrate in equatorial region of maturation spindle.

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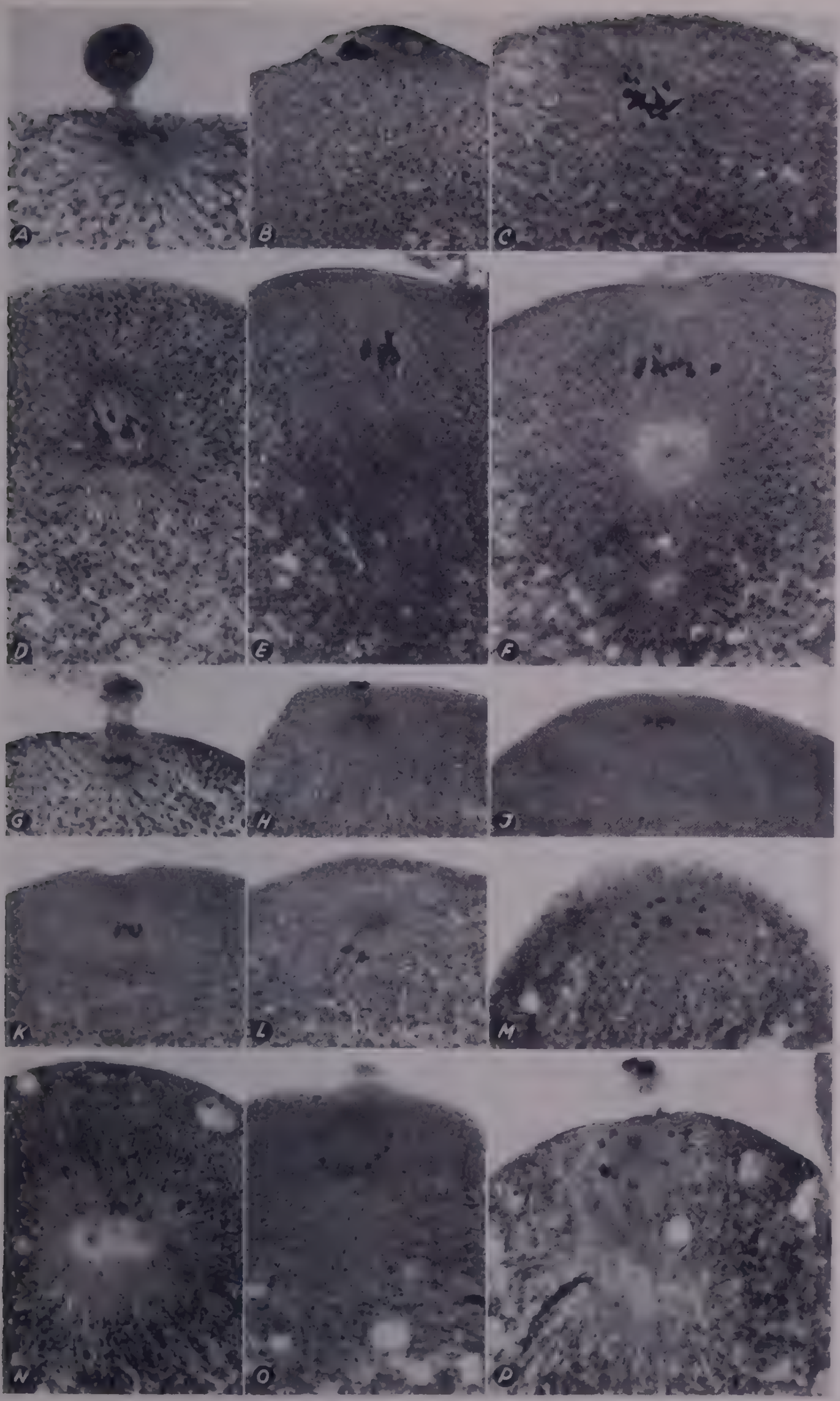

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Plate 1

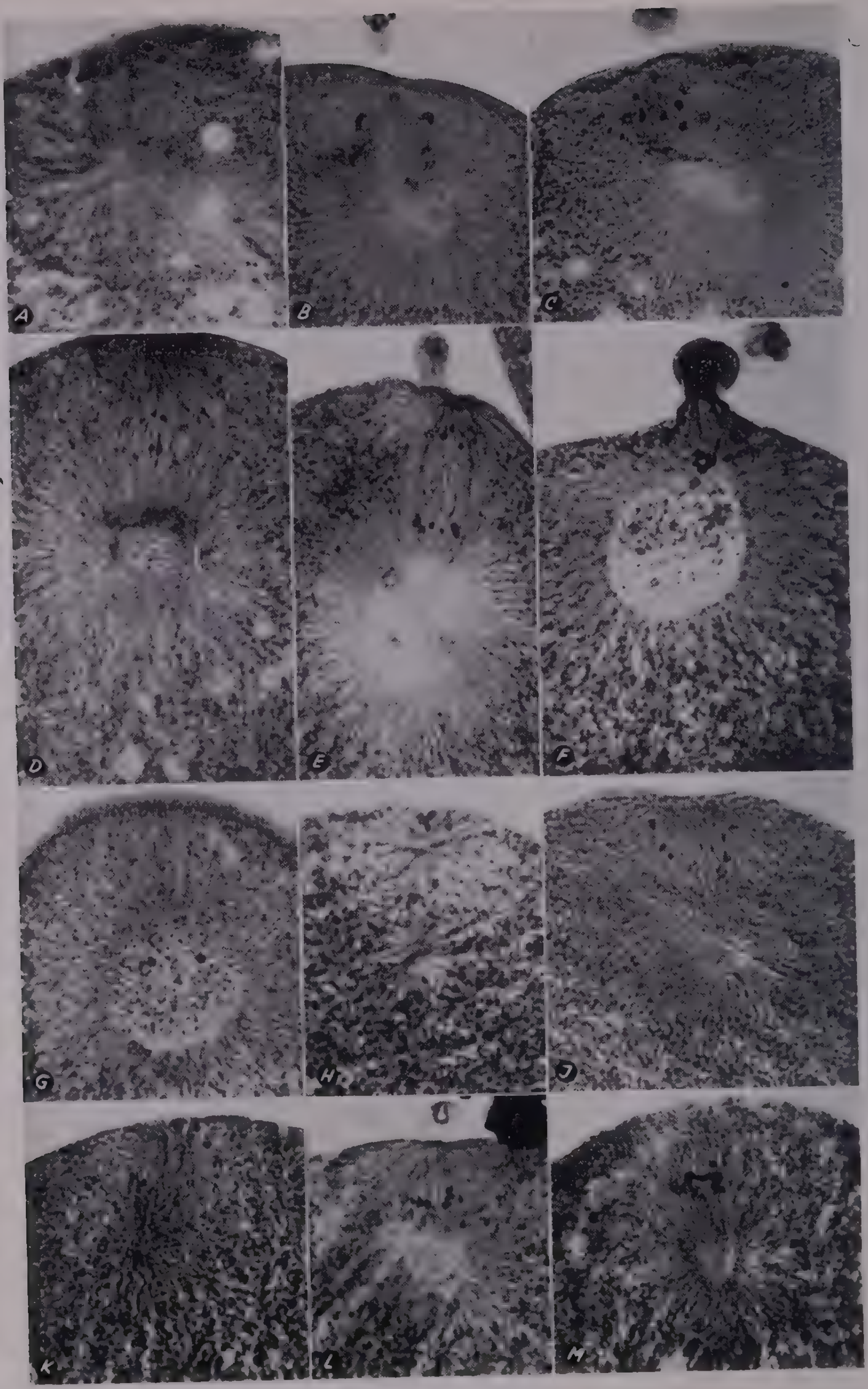

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Plate 2

The Pigment Cell System in the Light Sussex Fowl

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WITH TWO PLATES

INTRODUCTION

LIGHT SUSSEX embryos and day-old chicks have no pigmentation of their plumage. Some pigmentation appears at the bases of the wing and tail primaries during the first week after hatching, and at about 6 weeks each neck feather shows a broad longitudinal medial black stripe. The saddle, flank, and breast feathers remain unpigmented, except in some strains which may show some black flecks in these feathers as the bird ages. Thus in the adult Light Sussex the flight feathers of the wings and tail show considerable areas of black, the neck feathers have a broad black stripe on each side of the rachis, and the rest of the plumage is white (Plate 1, figs. A, B, C). The question therefore arises as to whether the pigment cells are present and non-functional, or absent, in the white feather of a partly pigmented breed such as the Light Sussex.

Hamilton (1940) made a study of the pigment cell systems of wholly white and of coloured breeds of fowl, and of various other species of bird. He showed that in tissue culture the back skin of embryos both of white and of coloured breeds produced melanocytes, but of different kinds. The melanocytes produced in the cultures from coloured breeds appeared to retain their functional activity and to remain expanded for long periods (up to 10 days). He further noticed that in the breeds whose melanocytes showed 'high viability' in culture (coloured breeds), apparently functional melanocytes were found much further distally in the growing feather germs of the adult bird; while in the breeds whose melanocytes showed 'low viability' in culture (white breeds), the few melanocytes found at the bases of the growing feather germs seemed to become 'senile' much more proximally. He thus concluded that the melanocyte of the coloured breed is better able to live and function in the environment found more distally in the feather, therefore lives longer as a functional melanocyte, and so donates more melanin. The melanocyte of the white breed, it is supposed, cannot tolerate the conditions found distally in the feather germ, and so becomes 'senile' relatively quickly before having donated sufficient melanin to give colour to the resulting

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feather. This theory of 'differential viability' of the melanocytes, however, would seem insufficient to account for the pigmentation of breeds such as the Light Sussex.

It has been shown by Lillie & Wang (1941, 1943, 1944) and by Wang (1943) that in some birds which show tract-specific differences of shape and colour pattern in their plumage, it is the epidermis which gives the melanoblast population its 'cues' as to which kinds of melanocytes to produce. They transplanted whole feather papillae in the Brown Leghorn and found that tract specificity as regards both shape and colour pattern was carried with them. They then transplanted only the dermal papillae, and found that these induced host region epidermis to produce feathers; these feathers were of host tract type. If some donor epidermis had been included in the graft, the resultant feather showed a chimerical structure, the coloration being also correlated very closely with the epidermal components. These experiments do not prove conclusively that the epidermis is the controlling tract-specific factor as regards pigmentation: the pigment cell system could itself possess regional specificity and be associated with this epidermis. However, it has been demonstrated by Willier & Rawles (1938, 1940) that the pigment cell stock does not carry tract-specific factors. These workers transplanted pieces of embryo skin into the wing-buds of 72-hour White Leghorn embryos and discovered by the examination of sectioned material that only the pigment cells of these pieces contributed to the plumage of the hosts. From whatever region in the donor bird these pieces had been taken, the colour pattern produced in the feathers of the host was always that consistent with these feathers. That the pigment of such feathers is in fact produced by the donor pigment cells has been clearly demonstrated by the experiments of Rawles (1939) in which American Robin was donor to White Leghorn. The pigment granules of these birds are very different in shape and it was observed that the granules of melanin in the pigmented feathers of the host White Leghorn were of donor Robin type.

Trinkaus (1948, 1952, 1953) has shown that, in the Brown Leghorn, the pigmentation of the plumage of the embryo and early chick does not respond to changes in thyroid hormone and oestrogen levels, as does that of the adult. He implanted tissue from growing feathers of adult Brown Leghorns, which do respond to such changes, into various sites under the skin of White Leghorn embryos. He showed that the pigment cell systems of these pieces, although taken from adult feather tract whose pigmentation does respond to hormone changes, do not respond to changes in hormone levels in their new site. He thus supposed that the pigment cell response to changes in hormone levels was mediated by the environment, probably by the epidermis, and that this mediation occurs only when the epidermis has become 'mature', and that 'immature' epidermis does not, or cannot, normally mediate in this way. These results seem hard to reconcile with those of Hamilton (1940), who showed that embryonic back skin of some other breeds grown *in vitro* produced more red melanocytes when oestradiol or testosterone propionate were added to the culture medium; the epidermis in these cultures was, of course, 'immature'. It is interesting in this respect to note that Trinkaus found testosterone to evoke no pigmentary response *in vivo*. Trinkaus further found that the time of attainment of 'maturity' in the feather tracts of the Brown Leghorn chick varied significantly from tract to tract, and that this 'maturity' of a given follicle could be hastened as compared with neighbouring follicles by repeated plucking of the young feathers developing from it. He concluded that the 'maturity' was not a general metabolic function but a change occurring independently in the history of individual feather germs. It may be noted in this connexion that in the Light Sussex the order in which the tracts acquire pigmentation is in many respects similar to the order of attainment of 'tract epidermis maturity' as found by Trinkaus in the Brown Leghorn.

The work described here is an attempt to discover the mechanisms involved in the production of unpigmented feather in a breed, the Light Sussex, some of whose pigment cells at least may contribute to the production of fully pigmented feathers. Tissue-culture experiments were used to determine whether or not competent pigment cell systems are present in the skin of the unpigmented embryo, and in the white feather areas of adult birds. Autograft experiments were used to demonstrate the role of the epidermis in determining the tract specificity of pigmentation in the plumage of the adult Light Sussex.

MATERIALS AND METHODS

Animals

Light Sussex, Brown Leghorn, Rhode Island Red, and Rhode Island Red × Light Sussex hybrid eggs were obtained from a local poultry breeder with the assurance that they came from pure stock. Some of each batch were always allowed to hatch, and all the chicks showed characteristics of the stated breeds. These eggs were used to provide the embryo extract for the *in vitro* studies, and the tissue for cultures of embryo skin.

Light Sussex hens were obtained from local suppliers as of pure stock and had no feather flecking over the saddle region. These birds were used in the papilla transplant series and as sources of feather collar tissue and venous plasma for the *in vitro* studies. Some plasma was also obtained from large cockerels, Rhode Island Red or Rhode Island Red × Light Sussex hybrids.

Tissue-culture methods

For the first few series of cultures the embryo extract was made up in the standard way (and was always used the same day). In later cultures the embryo was minced in its own blood, and in some cases no Tyrode was used to dilute the extract, the brei being centrifuged after grinding to a smooth cream. Unchilled arterial plasma was used in these later series.

Most of the cultures were hanging drops (or Maximow modifications of hanging drops, Maximow, 1929) and the cultures were all washed and fed and subcultured if necessary every 48 hours. Some watch-glass cultures were put up to provide material for sectioning and histological examination. The hanging drops were fixed and lightly stained with haematoxylin or malachite green on their coverslips.

Although normal precautions were taken to prevent infection of the cultures and of the media, a considerable number of the cultures were found to be infected, especially the cultures of feather material (as was to be expected). In all cases where the infection appeared to affect the growth or metabolism of the culture the culture was discarded irrespective of whether melanocytes had been produced in it or not. Antibiotics (penicillin and streptomycin) were used only in two of the earlier embryo back skin series, and no pigment cells were observed in any of these early series. No antibiotics were used in the subsequent culture media, although a mixture of penicillin and dimycin was used to wash the explant in the earlier feather series.

Preparation of feather tissue

A method was developed for the culture of feather collar explants. As the keratinized layer of growing feather germs is considerably infected, the problem arises of isolating pieces of the live tissue without risk of infection. The method finally adopted will now be described.

The pin-feather consists mostly of dead tissue; the outer layers are probably entirely dead. The feather was plucked through a pad of cotton-wool soaked in 75 per cent. alcohol and the basal centimetre or so was cut off into a large volume of sterile saline in a Petri dish (in the earlier experiments this contained antibiotic, but this was later omitted). This cylinder was then split along one generator with a hot razor blade and transferred to a perspex cover where it was opened out and pinned to a flat piece of sterile cork with the keratin face against the cork. The mesoderm was removed from the upper surface and kept in sterile saline, if required, in the manner usually employed in the making of standard split and flattened preparations of feather bases.

Explants could then be taken with needles from the exposed inner surface of the ectoderm. In all cases in this work explants were only taken from the inner ectodermal surface proximal to the visible formation of barb ridges, as it was felt that further distally the pigment cell system might well have been influenced by local environmental factors. It seems from examination of the feather collar cultures that pure ectodermal cultures are not as difficult to obtain as had been supposed; mesodermal elements seem to become recognizable, if present in the culture, at about 24 hours as a copious growth of fibroblasts (mechanocytes). In many of the feather collar cultures no such growth occurred and it was assumed that no mesoderm was present in these explants.

The transplantation of feather papillae

All transplants in the series were autografts. The follicles between which the transplants were to be made were marked either by tattooing or by pterylar identification from clipped feathers or tattoo marks, and the feathers growing in these chosen follicles were plucked and filed. When the subsequent pin-feathers had attained sufficient length for them to be plucked without undue damage to their papillae, these were plucked and the operation was performed substantially as described by Lillie & Wang (1941, 1943) except that in cases where the epidermal coat of the papilla was removed, this was dissected and not removed chemically. This operation was performed by pinning an attached flap of follicle wall to a piece of cork, incising the epidermal coat of the papilla longitudinally on several generators, and gently pulling the dermal papilla away, freeing it with cataract knives if necessary. Other operations on papillae were usually performed with fine iridectomy scissors. It was found to be unnecessary to bandage the operated areas as in most cases the bird did not recover completely from the anaesthetic for some hours, and by then the areas were quite dry and did not appear to inconvenience the birds at all; at no time were they observed to peck or scratch the areas, and on the few occasions when the birds were bandaged they appeared considerably inconvenienced.

RESULTS

Tissue culture

Embryo tissue. An attempt was made to repeat some of the work of Hamilton (1940) by culturing pieces of back skin of 7–8-day embryos of various breeds. In the early series no melanocytes developed in any of the cultures, even in those from fully pigmented breeds, e.g. the Brown Leghorn and the Rhode Island Red. The medium was then modified in various respects, and it was found that a medium containing arterial plasma which had not been chilled, and undiluted embryo extract, appeared to be most satisfactory for the development of melanocytes in these cultures.

In the course of inquiry into the most satisfactory medium for the growth of melanocytes *in vitro*, some Light Sussex embryo skin explants both from future pigmented (neck and wing) and from future non-pigmented (back and flank) areas produced melanocytes in some of the media tried. These appeared in many respects to be comparable with the Brown Leghorn and Rhode Island Red melanocytes produced in the same media.

In an attempt to discover the 'viability' of these Light Sussex melanocytes in culture, as compared with one of those breeds (Rhode Island Red) which Hamilton had shown to produce 'high viability' melanocytes in his cultures, several more series of cultures (about 100 in all) of Light Sussex 7-day embryo back skin, neck skin, and wing skin were set up. Thirty-one of these produced significant amounts of pigment in culture. Including those series used in estimating the suitability of the various media for pigment production *in vitro*, 236 cultures were put up from 40 Light Sussex embryos between 7 and 8 days old. Of these, 81 produced pigment; 46 of these cultures were of neck and wing skin, 35 were of saddle and flank skin (Plate 2, figs. G, H).

Very few of these melanocytes were observed to be in an expanded condition. This made any comparison of the 'viability' of the Light Sussex melanocytes with those of the pigmented breeds impossible.

Tissue from feather of adult birds. In order to discover whether there are cells capable of pigment production in the wholly white feather germs of adult Light Sussex, explants from the collar regions of growing wholly white saddle feathers were explanted. Comparable pieces of Brown Leghorn feathers and of the future pigmented parts of growing Light Sussex neck feathers were explanted to serve as controls in many but not in all series. Whenever pieces of the future black region of growing Light Sussex neck feathers were cultured to serve as controls for the white saddle feather material, some explants were also taken from the future unpigmented regions of the same neck feathers and cultured at the same time.

A total of 50 cultures was put up of Brown Leghorn saddle, neck, and breast pin-feather pieces, all taken from the collar regions of pin-feathers. In none of these pieces could melanocytes be seen at explantation. Twenty-eight of these cultures were subsequently found to be infected and were discarded. Of the remaining 22, 10 failed to produce pigment in culture. Of the 12 cultures which did produce melanocytes, 5 produced definitive feather structures. In 4 cultures the growth was apparently random and unorganized and these 4 cultures only produced few melanocytes. The other 3 cultures which produced melanocytes showed little or no growth, but some organization of the explant (encapsulation, &c.) could be observed in 2 of them. Almost all of the melanocytes produced in the cultures were black series, brown melanocytes being extremely rare, even in the cultures from saddle and neck feathers.

A total of 50 cultures was made from Light Sussex saddle and neck pin-feather collar. In none of these pieces could melanocytes be seen at explantation. Two of these cultures were subsequently found to be infected and were discarded. Of 32 saddle (white) pin-feather collar region cultures, 21 failed to produce pigment in culture. Of the 11 cultures which produced melanocytes, only 1 showed no development of barb ridges. The others all developed areas of barb ridges in explant or outgrowth or both. In these cultures the melanocytes appeared mainly along the barb ridges and varied considerably in number and melanin content between cultures and even between contiguous barb ridges.

Plate 2, fig. K, shows an explant from the collar region of a wholly white saddle feather which has been cultured for 84 hours. Barb ridges may be seen to have developed, and many melanocytes have developed in association with these. Many of the ectodermal cells contain melanin which has presumably been donated by these melanocytes (see also Plate 2, fig. L).

Two Light Sussex neck pin-feathers were used in the above series as sources for 16 explants. Nine of these explants were taken from the future black sectors of the collars, 1 was taken from about the margin of the future black and the future white sectors, and 6 were taken from the future white sectors. Two of the

9 from future black sectors keratinized early, the other 7 all produced various amounts of pigment, both in the barb ridge areas and to some extent in other parts of the cultures. The explant derived from the margin of the future black and the future white sectors keratinized after having formed a little pigment. Of the 6 explants from the future white sectors, 4 failed to produce any pigment in culture and the other 2 appeared similar to the dorsal sector explants in that melanocytes appeared in the areas of barb formation.

In an attempt to learn more of those differences between the dorsal and ventral parts of the collar of Light Sussex neck feathers which cause the difference in pigmentation, a further series of neck feather explants was made and the location of the explants in the collars before explantation was more precisely noted. From each of 3 pin-feathers, explants were taken from the mid-dorsal region, from areas to either side of the mid-dorsal region, from the mid-ventral region, and from areas to either side of the mid-ventral region. Two explants were taken from each of the above sectors, 1 from the proximal end of the collar, and 1 from the distal part of the collar region just proximal to the visible commencement of the barb ridges. There was thus a total of 36 explants. Explants from the same locality in every case showed similar results except, of course, in cases of infection.

TABLE 1

Behaviour of some collar explants from 3 Light Sussex neck pin-feathers grown in vitro

Distal collar	*Feather 1*	*Feather 2*	*Feather 3*
Mid-ventral	Keratinized	Keratinized	Infected
Lateral ventral	Keratinized	Infected	Infected
Lateral ventral	Keratinized (some melanin)	Keratinized	Infected
Mid-dorsal	Infected	Random growth (no melanin)	Random growth (no melanin)
Lateral dorsal	Keratinized (some melanin)	Keratinized (some melanin)	Keratinized (some melanin)
Lateral dorsal	Random growth (no melanin)	Keratinized (some melanin)	Random growth (no melanin)

Proximal collar	*Feather 1*	*Feather 2*	*Feather 3*
Mid-ventral	Barbs and melanocytes	Barbs and melanocytes	Barbs and melanocytes
Lateral ventral	Barbs and melanocytes	Barbs and melanocytes	Barbs and melanocytes
Lateral ventral	Keratinized (some melanin)	Keratinized (some melanin)	Barbs and melanocytes
Mid-dorsal	Infected	Keratinized (no melanin)	Random growth (no melanin)
Lateral dorsal	Infected	Barbs and melanocytes	Barbs and melanocytes
Lateral dorsal	Infected	Barbs and melanocytes	Late keratinization (some melanin)

Table 1 shows the results of this series. It will be seen that the explants from ventral and distal collar keratinized early, and of these only one showed any pigmentation. Of those explants from dorsal and distal collar, the 4 which showed random growth produced no melanin and 4 others keratinized after having produced some melanin. Of those explants from proximal collar, all those from the ventral (future white) part produced melanin (see Plate 2, fig. J); only two of these keratinized, and the others all showed melanocytes lying along barb ridges. Of the dorsally derived explants (future black) two of the uninfected cultures did not produce any melanin; 3 showed barb formation and melanocytes (see Plate 2, fig. I).

The above results on the culture of explants from Light Sussex white-feather areas (white saddle feathers and the white regions of the neck feathers) demonstrate that a competent pigment cell system is present in the collar region from which such white feather is formed, as well as in the collar regions from which pigmented feather is formed, and may be demonstrated in certain culture media.

Papilla transplants

Several series of papilla transplants were performed between saddle and breast follicles of Brown Leghorn capons. Some of these papillae were stripped of their epidermis. The results obtained with a total of 32 feathers over three feather generations agreed with those of Lillie & Wang (1941, 1943) in all respects.

On three Light Sussex hens, a total of 60 feathers was produced from neck, saddle, and flank follicles which had received implanted papillae after their own papillae had been removed. In all cases where the donor papillae were implanted intact, the first generation feathers were of donor tract structure and pigmentation. Subsequent feathers from some of these follicles tended to show areas of host structure, always associated with host-type pigmentation.

In all those cases in which papillae which had been stripped of their epidermal coats were implanted, feathers partly or completely of host-tract structure and pigmentation were produced. Other chimerical feathers were produced in some cases where papillae thought to be intact had been implanted, in the second and subsequent generations. On one bird, 3 neck follicles produced neck feathers of normal structure and colour after having produced normal donor-tract feathers (unpigmented flank feathers).

Fifteen structurally normal neck feathers were produced in saddle follicles and 7 in flank follicles, over three feather generations. All were normally pigmented, despite the fact that previous feathers from these follicles had been, of course, white. Eleven normal unpigmented saddle feathers and 3 normal unpigmented flank feathers were produced in neck follicles. Six unpigmented saddle feathers were produced from saddle follicles which had received stripped neck papillae. Thirteen neck feathers were produced from neck follicles which had received saddle or flank papillae after their own papillae had been removed, over four generations; all were normally pigmented (see Plate 1, figs. D, E).

Five feathers of chimerical structure were observed. In 4 of these the character of the pigmentation was closely correlated with the pattern of chimerical structure as determined by the morphology of barbs and barbules. In the other, a vestigial feather, precise structural analysis proved to be impossible.

Of all of these feathers, with the possible exception of the vestigial chimera, it may be said that all pigmented feather was of neck-feather structure, and that all feather of saddle structure was unpigmented; feather of neck structure which was unpigmented was, of course, also found (normal neck feathers have considerable areas of white). Thus it may be concluded that the pigmentation character is not correlated with the tissue environment of the papilla, but solely with the derivation of the *epidermal* component of this papilla.

DISCUSSION

The tissue-culture experiments have demonstrated that the pigment cell system in the white plumage of the Light Sussex embryo and in some of the white plumage areas of the Light Sussex adult is capable, under certain conditions, of melanin production. In certain culture media the production of melanocytes in these tissues appears to proceed in a manner comparable with the production *in vitro* of melanocytes by Brown Leghorn or Rhode Island Red tissue. The important difference in respect of melanocyte production between *in vivo* and *in vitro* conditions is very difficult to assess. That it involves a relationship between the pigment cell system and other tissues of the animal seems certain, as these experiments have shown little or no difference between the pigment cells which would have produced pigment *in vivo* (in neck-feather dorsal collar) and pigment cells from white feather areas (saddle-feather collar and neck-feather ventral collar) in respect of their production of pigment *in vitro*; that is to say, the pigment cell system of the saddle feathers in the Light Sussex adult appears to be capable of the production of normal melanocytes in the same way as is the pigment cell system of the black parts of the black and white neck feathers.

In some of these white-feather collar cultures the growth was entirely epitheliocyte in nature, it being inferred that no mesoderm was present in the culture. In many of these cases melanocytes appeared in the developing barb ridges (see Plate 2, figs. K, L). It must therefore be supposed that there were melanoblasts or non-pigmentary dendritic cells already in this ectodermal tissue; if the tissue had been left in the feather these would not have developed melanin, but in the culture conditions they did produce, and in some cases donated, melanin. 'Espinasse (unpublished) has noted that melanocytes may be found in the mesoderm of the future white regions of Light Sussex feathers but only very rarely in the ectoderm of these regions. From the fact that melanocytes do appear in this ectoderm from future white feather in culture, it seems likely that it is the relationship between this ectoderm and these pigment cells which is of such a kind that pigment production does not occur *in vivo*. However, by modifying this relationship (e.g. in certain culture media) a situation is brought about in which melanin production can and does occur. Further, some of the explants from future pigmented region of neck-feather collar did *not* produce pigment *in vitro* as they would have done *in vivo*; thus some culture conditions may so disturb the relationship between the pigment cell system and the future pigmented ectoderm that pigment formation is *prevented*, although the medium appears to be adequate for the maintenance, growth, and differentiation of the culture. It seems, therefore, that both in the case of the pigmented feather of the Light Sussex and in that of the non-pigmented feather of the Light Sussex the pigmentary response may be reversed (*in vitro*) while differentiation and growth of the feather barb system remains apparently unimpaired.

It has been deduced from the work of Willier & Rawles and of Wang (see introduction) that the epidermis of a given feather germ gives a series of 'cues' to the underlying pigment cell population, to which this pigment cell population responds according to its nature by the production of a pigmentation consistent with the 'cues'. (Hardesty, 1933, has envisaged a 'genetically determined stencil'.) Hamilton has demonstrated that the pigment cell system of some wholly white breeds of fowl is apparently incapable of the production of pigmented feather. These melanocytes respond to *all* 'cues' by the production of insufficient pigment to give noticeable colour to the feathers concerned. The pigment cell system of a fully pigmented breed (e.g. Brown Leghorn), on the other hand, responds to 'cues' from the ectoderm of all kinds of normal feather germs by the production of melanocytes which *do* produce enough melanin to colour the feather. The Light Sussex pigment cell system appears to respond to certain situations in the bird, and to certain environmental conditions in culture, by producing pigment, and to others by not producing pigment. This may well be comparable with the situation in the Brown Leghorn where the pigment cell system responds to certain 'cues' by the production of brown melanin and to others by the production of black melanin. We know that few or no brown melanocytes are found in the Light Sussex. In fact the Light Sussex pigment cell seems to behave *in vitro* and *in vivo* in many respects like a Brown Leghorn pigment cell which cannot produce brown pigment.

The pigmentary situation in the Light Sussex embryo and early chick, however, has yet to be considered. Trinkaus has shown that 'immature' epidermis does not mediate the response of the pigment cell system of the Brown Leghorn embryo to changes in hormone level, as does 'mature' epidermis. It seems at least likely that the situation in the Light Sussex embryo and early chick is related to this situation in the Brown Leghorn. Further, although Trinkaus obtained no pigmentary response to thyroxine or oestrogens in the Brown Leghorn chick, Hamilton stated that more red-brown series pigment cells were produced in his embryo back skin cultures in the presence of sex hormones. Thus it seems that the effect of the 'immaturity' of the epidermis did not occur in his tissue cultures, either because the pigment cell system responds to some extent directly to the hormones *in vitro* (cf. Markert, 1948) or because the epidermis/pigment cell relationship is modified in such cultures and mediation may occur. In view of these results it seems that the production of pigment *in vitro* by Light Sussex embryo back skin is in some way related to the modification in pigmentary response of Hamilton's back-skin cultures. The situation in the Light Sussex embryo and early chick, where insignificant amounts of pigment are produced, would then be comparable with that found in Brown Leghorn embryos and early chicks by Trinkaus, where the pigmentary response to changes in hormone level is not mediated.

The transplantation results complement those obtained in the *in vitro* studies. In no case was feather produced from neck-type ectoderm (as evinced by the structure) which did not have the characteristic neck-feather pigmentation; nor was any feather of saddle structure produced which was significantly pigmented. It is very probable (from the results of the transplants between areas of different genotypes (Cock & Cohen, 1958) that at least some of these feathers of neck structure and pigmentation growing in saddle—as described in the present work—were using the local pigment cell system which would have failed to pigment saddle feathers, and that at least some of the feathers of saddle or flank structure and pigmentation growing in neck follicles were using the local pigment cells, which failed to colour them. Further, the experiments with dermal papillae using local epidermis to form feathers all produced feathers of host-region type both with regard to shape and pigmentation, and the few chimeras that were produced in the experimental series all showed correlation between pigmentation type and structure of all parts of the feathers, as far as could be judged. (In one chimera the neck portion of the feather was totally black, but the saddle portion of this feather was quite white; see Plate 1, fig. F.) It seems therefore that the relationship between dorsal region of Light Sussex neck feather and Light Sussex pigment cells either from pigmented or from white regions always gives pigmented feather, while the relationship between saddle or flank feather epidermis and pigment cells from white or from pigmented regions always gives unpigmented feather. This confirms the assumption, made on the basis of the tissue culture experiments, that the Light Sussex pigment cell system does not carry tract-specific factors but that any Light Sussex pigment cell system may produce either white or pigmented feather according to the nature of its 'cues'. The 'cues' which it receives in tissue culture tend to cause melanocyte production (or to remove an inhibition of melanocyte production), as do those which it receives in the dorsal region of the neck-feather collar, while in the saddle and flank regions of the bird the relationship is such that very few melanocytes are produced.

It is now generally accepted that the character of the pigmentation of any tissue or organ is the result of such an interaction between the pigment cell system and its environment in that situation. In almost all cases in which the problem has been investigated it has been found that the melanoblast system is potentially homogeneous throughout the tissues of the animal, the difference in response leading to different kinds of pigmentation being a result of the relationship between the pigment cell system and its immediate environment. The implantation experiments of Willier & Rawles have demonstrated that this is the case for many breeds of fowl, and Markert & Silvers (1956) have postulated a similar situation in the mouse as a result of microscopic examination of the tissues. However, some situations are generally regarded as showing a differentiation of the pigment cell stock into a heterogeneous system. Trinkaus has demonstrated that the pigment cells of adult Brown Leghorns behave differently, in the coelomic epithelium of host embryos, from the pigment cells of embryo Brown Leghorns in the same situation, thus indicating a temporal change in the pigment cell stock of this breed. Pepper (1956) considered as a result of skin-grafting experiments that the white areas of the Hooded Rat do *not* contain a system capable of pigment production, while the black areas do, of course, contain such a system. This would be an example of a spatial differentiation of the pigment cell system similar to that which was, at one time, believed to occur in the Light Sussex fowl. Silvers (1956 *a*, *b*, 1957) maintained that no competent pigment cell system is present in the white regions of recessively spotted mammals.

The tissue-culture work described here has established that the pigment cell system of both white and pigmented feather of the Light Sussex adult, and the feather germs of the Light Sussex embryo, are capable of pigment production in some circumstances. The results of papilla transplantation experiments on adult birds makes it seem very likely that the pigment cell system of the plumage of the Light Sussex is indeed a homogeneous system in respect of its capability of pigment production, and that local differences in pigmentation are dependent on the interaction of this pigment cell system with another system which shows local variation, almost certainly the epidermis. This is a situation comparable with that found in the House Mouse by Markert & Silvers and in other breeds of fowl by Willier & Rawles.

SUMMARY

1. The pigmentation pattern of the Light Sussex fowl is described and considered as a case of ‘partial pigmentation’.

2. By the use of tissue-culture methods it has been demonstrated that a competent pigment cell system occurs in the white plumage of the Light Sussex embryo and adult.

3. Papilla transplantation experiments were used to evaluate the role of the epidermis in determining the fate of these pigment cells. The results obtained indicate strongly that the epidermis determines whether or not the equipotential pigment cells shall or shall not produce pigment *in vivo*.

4. It is concluded that the pigment cell system of the Light Sussex responds to certain conditions *in vivo* and *in vitro* by the production of sufficient pigment

to colour feather, and to other conditions by the production of insufficient pigment.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. A group of day-old Light Sussex chicks. No pigmented feather is visible.

FIG. B. Six-day Light Sussex chicks. Pigmented patches have grown out in the wing primaries of some specimens.

FIG. C. An adult Light Sussex hen.

FIG. D. Five feathers growing from neck feather papillae implanted into saddle follicles, third generation. *Upper Right to Lower Left*: *a*, an intact papilla was implanted: a series of three neck feathers of reversed orientation was produced; *b*, a stripped (dermal) papilla was implanted: two saddle feathers of slightly abnormal structure were produced after a 'resting' period, both rotated 90° as compared with previous feathers from the follicles; *c*, *d*, *e*, three intact papillae were implanted; each follicle produced three neck feathers: *c*, with normal orientation; *d* & *e*, with orientation reversed.

FIG. E. Five feathers produced by neck feather papillae in saddle follicles, first generation. *Left to Right*: *a*, the neck papilla was stripped of its ectoderm and bisected sagittally before implantation; the feather is split, saddle type; *b*, the neck papilla was stripped before implantation; the feather is a vestigial chimaera with black and white barbs, whose structure could not be determined; *c*, the neck papilla was bisected sagittally before implantation; the feather is double, neck type; *d*, *e*, the neck papillae had their apices removed before implantation; the feathers have defective apices and are of neck type.

FIG. F. A feather of chimerical structure produced in a neck follicle by a saddle papilla the ectoderm of which had been removed only dorsally before implantation. It was determined by examination of barb structure that the white area is saddle type; the more distal part of the black area is of neck type, while the proximal part of the feather is abnormal in structure, and can be referred neither to neck nor saddle.

PLATE 2

FIG. G. Part of a hanging drop culture of Light Sussex embryo back skin, fixed at 96 hours. Melanocytes have developed in the growing feather germs. Light background staining. ×83.

FIG. H. Part of another hanging drop culture of Light Sussex embryo back skin, fixed at 96 hours. Melanocytes have appeared diffusely through the tissue. Light background staining. ×50.

FIG. I. Part of a hanging drop culture of lateral dorsal collar, proximally, from a Light Sussex neck feather fixed at 96 hours. No melanocytes were visible at explantation. Deeply pigmented barbs may be seen, and some scattered melanocytes. The culture was in process of keratinization. Light background staining. ×66.

FIG. J. A hanging drop culture of mid-ventral collar, proximally, from the same feather fixed at 48 hours. No melanocytes were visible at explantation. A 'ventral triangle' has developed and melanocytes have appeared. Light background staining. ×66.

FIG. K. Part of a hanging drop culture of Light Sussex saddle feather collar, fixed at 84 hours. No melanocytes were visible at explantation. Barb ridges have appeared in culture and melanocytes have developed in association with them and have donated pigment. Light background staining. ×83.

FIG. L. Part of another hanging drop culture of the same feather collar, fixed at 84 hours. No melanocytes were visible at explantation. Barb ridges have again appeared, and the melanocytes which have developed along them have donated considerable melanin. Light background staining. ×200.

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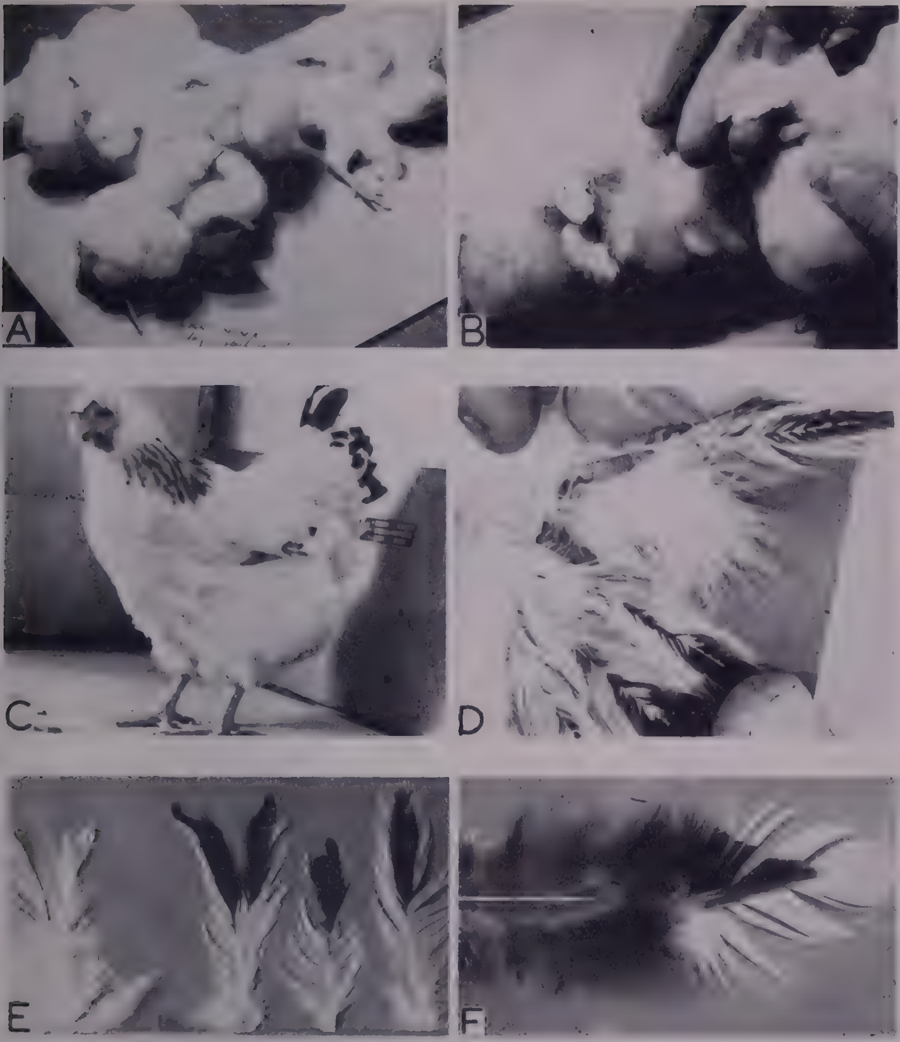
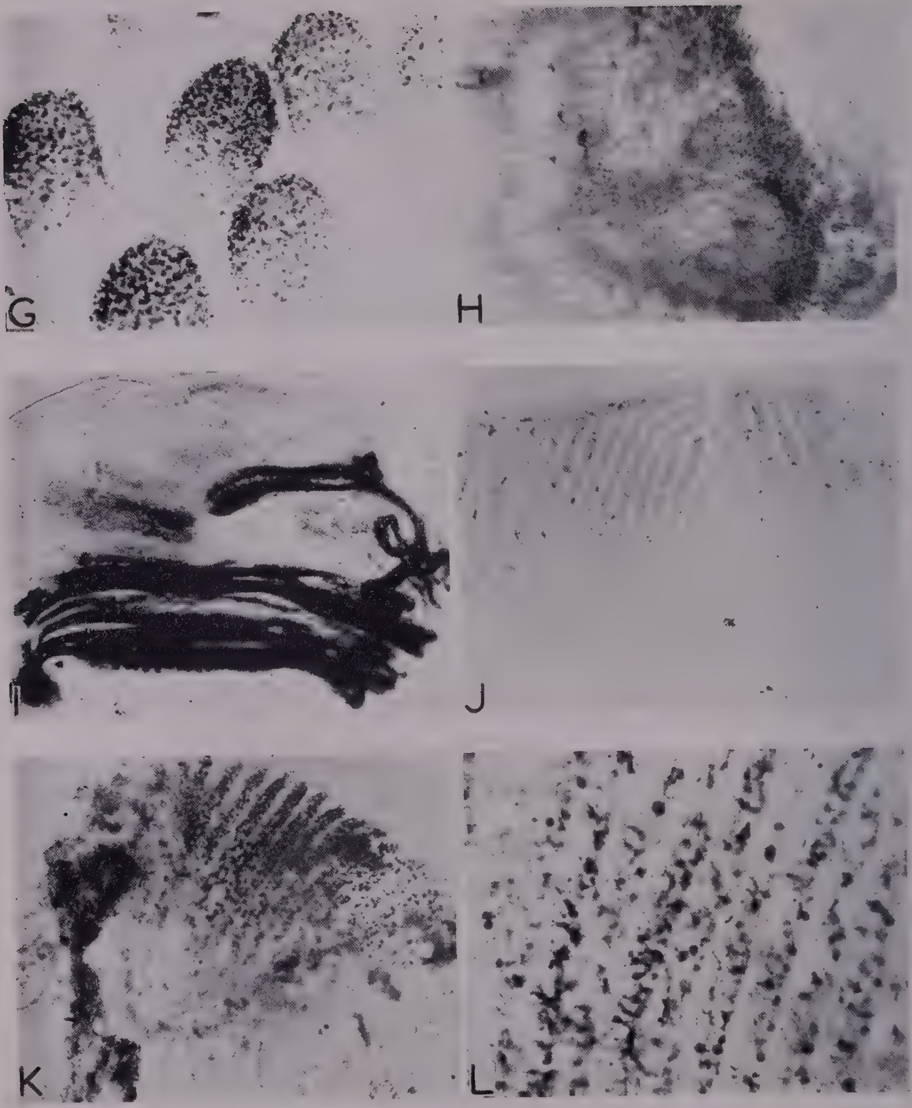


J. COHEN

Plate 1



J. COHEN

Plate 2

The Apparent 'Corneal Specificity' of Sensory Neurons

by GEORGE SZÉKELY[1]

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WITH ONE PLATE

INTRODUCTION

SINCE the first fundamental papers of Paul Weiss (1924), a considerable body of evidence has been accumulated in favour of the assumption that different tissues or organs transplanted heterotopically exert some specific modulatory influence on neurons, the processes of which happened to grow into the grafts; the grafts in turn provide these neurons with capacities present in the neurons normally supplying these tissues or organs. These observations led to the formulation of a 'resonance' hypothesis, but later Weiss himself elaborated a somewhat more cautious concept of 'specific modulation' (see Weiss, 1955), which was corroborated and extended in masterly manner in different fields of neurogenesis by Sperry (1951) and his collaborators. Strong support was given to this concept by an experiment of Weiss (1942) in which, by tactile stimulation of the cornea of a supernumerary eye implanted into the region of the nostrils or the ear capsule, an ordinary corneal reflex response could be elicited in the ipsilateral eye of the host. The cornea of the implanted eye being innervated by the trigeminus like that of the normal eye, this experiment was not completely convincing. But when Kollros (1943) implanted supernumerary eyes between the ear capsule and the base of the gill, a region innervated by the vagal nerve, stimulation of the cornea yielded also corneal reflexes of the host's ipsilateral eye. Thus the assumption of a 'corneal specificity' appeared to be well justified.

Working for other purposes with limbs of urodele larvae transplanted to the base of the gill, it happened that the distal part of an implanted limb was bitten off by another animal. Mechanical stimulation of the regeneration blastema evoked a corneal reflex of the ipsilateral eye, an effect never met with when an intact implanted limb was stimulated. This observation being inconsistent with the assumption of a 'corneal specificity' the matter was more closely investigated, and the results will be presented here.

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METHODS

The experiments reported were performed on larvae of *Pleurodeles waltlii* and *Triturus vulgaris*. The eye excised previously from young larvae was transplanted into the immediate neighbourhood of the base of the gills in larvae of similar age. In a second group a forelimb was transplanted into the same locus. A fortnight after the transplantation the grafts were tested by touch stimuli to discover whether they had received their sensory supply. After the mid-larval stage animals were kept in aquaria and fed maximally in order to promote optimum growth. They were investigated weekly for reflexes to touch stimulation, applied to the grafts by means of a fine glass needle. All stimulations and observations were performed under the low power of a stereoscopic microscope. Stimulation of larvae was made under water and of adults in air. A few days before the onset of metamorphosis the foot of the limb grafts was cut off and the animals were repeatedly tested as to the reflexes evoked by tactile stimulation of the resulting blastemata.

RESULTS

Responses from the cornea of supernumerary eyeballs

Seven larvae of *Pleurodeles* and three of *Triturus* had a supernumerary eye grafted homoplastically just at the base of the gills. The grafts developed normally; they were as large as the host's own eye (Plate, figs. A, B). The corneae remained perfectly transparent and they were reinnervated within one or two weeks of transplantation. This was determined by touch stimuli, the response to which was either the depression of the gills or an escaping movement. The gill depression response was already noticed in the first half of larval life, i.e. in the period in which this response can be elicited exclusively by stimulation of the vagal sensory area (Székely, 1959). At the onset of metamorphosis the orderly appearance of the corneal reflex to the stimulation of the graft developed in exactly the way described by Kollros (1942). Six *Pleurodeles* and one *Triturus* regularly yielded corneal reflexes from the grafted cornea until one year after metamorphosis. None of these animals showed any sign of a corneal response to stimulation of the skin around the grafts or of the corresponding cutaneous regions on the opposite side of the head.

Responses from the limb grafts

Eleven *Pleurodeles* and four *Triturus* had a forelimb graft transplanted homoplastically quite near to the base of the gills. The grafts developed normally and were reinnervated both by sensory and motor fibres of the 10th nerve. Touch stimulation applied to the limb grafts resulted in depression of the gills and a synchronous movement of the graft. Stronger stimuli caused either a head-jerk or an escaping movement, but never any specific responses of the host limbs. Any responses of the host limbs turned out to be either vestibular effects due to tilting of the animal during stimulation, or stepping movements preliminary to escaping reactions. Shortly before the onset of gill reduction the foot was cut off. Within a week a blastema developed on the tip of the grafts (Plate, fig. C) and at the same time the corneal reflex to mechanical stimulation of the host eye appeared. Touching the regeneration blastema of the foot very lightly with a fine glass needle, we were able to record in this period a complete corneal reflex in nine *Pleurodeles* and in one *Triturus*. The stimulation of the limb skin immediately next to the blastema proved completely ineffective in evoking any corneal reflex. Similarly, the stimulation of the skin of the head on the opposite side did not evoke any lid-closure reflex. The further the regeneration of the foot advanced, the higher became the threshold of the blastema for the corneal reflex. After complete regeneration of the foot, i.e. when all the four digits had appeared and the pigment pattern of the skin had become normal (Plate, fig. D), the corneal reflex disappeared. Even the strongest stimuli were ineffective, resulting only in the generally observed head-jerk or in escaping movements, but never in a corneal reflex or in an isolated response of the host's limbs. After reamputation of the foot and stimulation of the resulting blastema, the corneal reflex reappeared. The reamputation was performed in this case one month after metamorphosis. The development of the blastema and also the regeneration was delayed, and the threshold appeared to be somewhat higher than in the first case. One animal even displayed a corneal reflex from the blastema after the third amputation. From the fourth month after metamorphosis no corneal reflex from the resulting blastema was ever seen, and it may be mentioned that the regeneration of the foot also either failed or was incomplete after this period.

DISCUSSION

These experiments show clearly that tactile stimulation of a grafted cornea and of a limb regeneration blastema equally yield retraction of the host's eye on the same side. Cornea and limb blastema evidently cannot have any common 'specific biochemical properties', and consequently cannot exert the same effect of 'specific modulation' on vagal sensory neurons in the sense of Weiss's concept. But consideration of the histological type of innervation, both of the corneae and of the blastemata, may perhaps suggest an explanation of the foregoing results from another point of view. The sensory fibres of the cornea are known to form a meshwork-like plexus in the connective tissue of the corneal *substantia propria*, from which the free beaded terminals supplying the epithelium arborize. This is a nerve-ending of a low grade of differentiation subserving pain alone (Nafe & Wagoner, 1937) or, at most, pain and touch (Jalavisto, Orma, & Tawast, 1951). The sensory fibres in the blastema first form very similar terminal patterns of freely arborizing fibres, from which the characteristically more elaborate, partly encapsulated, and more highly differentiated receptors only gradually emerge during regeneration. Since the capacity of vagal sensory fibres supplying a limb regeneration blastema to bring about corneal reflexes lasts for as long as their nerve-terminals are of a low grade of differentiation, similar to that of the cornea, and is lost when the regeneration approaches the restitution of an ordinary limb, the explanation is close at hand: that certain little differentiated sensory terminal patterns of the head region may have the fundamental capacity to elicit corneal reflexes. A simple injury effect can be ruled out in view of the fact that from the stump of a foot recently cut off no similar reflexes can be provoked.

This explanation presupposes the existence of certain cues in the central nervous system, which—in spite of our familiar concepts concerning the function of fixed neuronal pathways—would be capable of interpreting the different peripheral messages. We may think here of the frequency code, so lucidly discussed in Granit's (1956) monograph: e.g. different conduction velocities and relative spike sizes due to different fibre diameters (Gasser, 1946); the sensitivity optima and the pattern of firing of thermoreceptors at different temperatures (Dodt & Zotterman, 1952); the so-called spike frequency / time differential of the retina elements which varies in specific fashion with wavelength (Donner, 1950). All these provide examples of the central interpretation of frequency codes. On the basis of modern neurophysiological knowledge of sensory discrimination and integration, it can be well imagined that the central nervous system has the capacity to analyse the different impulse patterns and to dispatch them towards different channels. The change of a given receptive field may alter the respective impulse pattern resulting in changed reflex relations. We cannot, however, completely neglect another more simple alternative, that sensory inputs arriving from nerve-terminals of primitive type could have the tendency to become generalized in the newt medulla, with the final result that the effector mechanism of the corneal reflex may be brought into action as an especially sensitive system for generalized states of excitation. This, however, would be only a marginal case of the previously discussed discrimination mechanism.

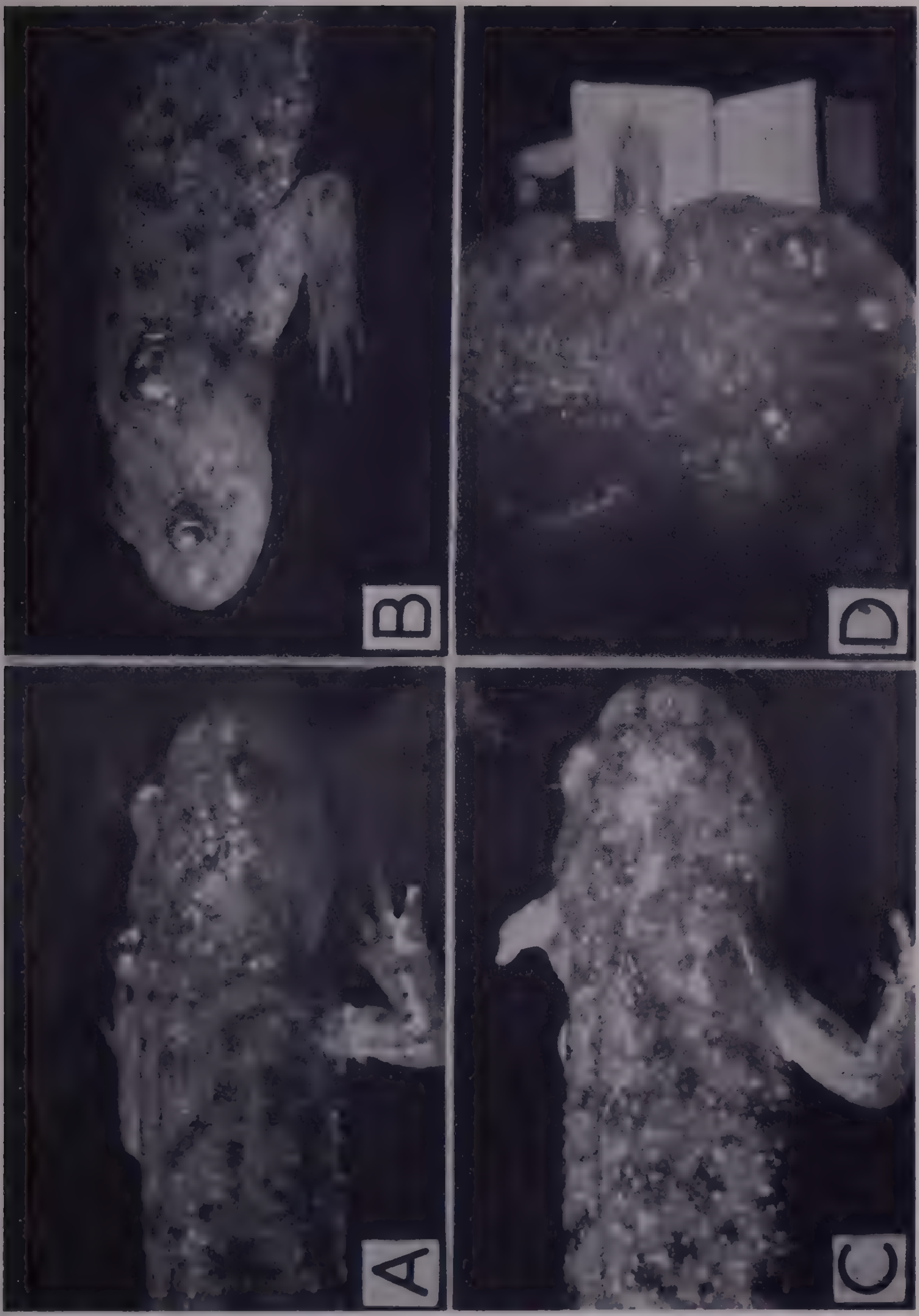
The strange fact, that stimulation of the grafted limbs innervated by the vagal nerve do not yield specific reflexes of the host's limb, while limbs grafted into the back of tadpoles (Miner, 1951) or into the dorsal fin of newts (Székely & Szentágothai, 1959)—even in non-limb segments—readily elicit such responses, may also call in question the current concepts of 'limb specificity'.

SUMMARY

1. Tactile stimulation of a forelimb grafted into the gill region in urodeles (*Pleurodeles waltlii* and *Triturus vulgaris*) yields no corneal reflex.

2. If the foot of such a grafted limb has been cut off, stimulation of the resulting regeneration blastema evokes a corneal reflex in the same way as does stimulation of the cornea of an eye transplanted into this region.

3. After complete regeneration of the foot the corneal reflex disappears, but



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from a new regeneration blastema developing after a second and in some cases even after a third amputation the corneal reflex can again be elicited.

4. These results are considered to be inconsistent with the 'modulation theory' of Weiss.

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EXPLANATION OF PLATE

Pleurodeles waltlii at the onset of metamorphosis.

FIGS. A and B. Eye-grafts in the posterior head region (at the base of the gills).

FIG. C. Forelimb grafted into gill region, the foot having previously been cut off. Stimulation of the regeneration blastema yields an ipsilateral corneal reflex.

FIG. D. The same animal one month later. No corneal reflex evoked by stimulation of the regenerated foot.

(Manuscript received 2 : iii : 59)

Recherches sur quelques aspects de l'hétéroploïdie expérimentale chez le Triton *Pleurodeles waltlii* Michah.

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AVEC TROIS PLANCHES

INTRODUCTION

DEPUIS les travaux initiaux de Fankhauser & Griffiths (1939), de Griffiths (1941) tirant parti des expériences de Rostand (1933), il est connu que l'hétéroploïdie peut être déclenchée chez les Amphibiens à la suite d'un choc thermique appliqué à l'œuf fraîchement pondu. L'œuf pris juste après la ponte et mis au froid (+1° / +3° C.) pendant cinq à vingt-quatre heures, n'émet pas son second globule polaire (Fankhauser & Godwin, 1948). Il reste ainsi dans le cytoplasme deux pronuclei femelles et un pronucleus mâle qui forment lors de l'amphimixie un noyau triploïde. Sur ce mécanisme fondamental se greffent diverses anomalies moins clairement analysées conduisant à des aneuploïdes. Certains germes enfin demeurent diploïdes. Les faits ont été étendus à diverses espèces d'Urodèles et d'Anoures (rev. par Fankhauser, 1945; Gallien, 1953).

Nous avons reproduit chez un Triton européen, *Pleurodeles waltlii*, les faits connus (Gallien & Mugard, 1950), les études poursuivies étant, dans nos projets, en relation avec nos recherches antérieures sur la détermination du sexe des Amphibiens. Cette question fera l'objet d'une publication particulière. Etant donné la durée des élevages et des expériences de croisement nous nous proposons de présenter un ensemble de résultats généraux déjà acquis. En effet au cours des recherches nous avons introduit une nouvelle méthode permettant l'appréciation indirecte de l'hétéroploïdie. Celle-ci, basée sur l'imprégnation argentique des extrémités caudales des larves, permet d'obtenir les limites cellulaires définies de l'épithélium (Gallien & Mugard, 1950). Par ailleurs l'application de la technique des parabioses a rendu possible l'élevage d'embryons hypomorphes, haploïdes ou aneuploïdes (Gallien, 1957).

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MATÉRIEL ET TECHNIQUES

Le Pleurodèle présente sur les autres Tritons l'avantage de pouvoir donner, au moins de septembre à mai, des pontes naturelles abondantes (Gallien, 1952), ce qui permet d'exclure l'incidence de l'hypo- ou de l'hypermaturité sur les germes observés. Lorsqu'une femelle a commencé à pondre, les œufs sont collectés toutes les 10 minutes et placés immédiatement dans l'eau froide (+1° / +3° C.). Après 5 à 20 heures, selon les expériences, les œufs sont transférés dans de l'eau à 18° C. où le développement s'achève. Une étude des phases de la fécondation normale menée parallèlement a montré que celles-ci durent 6 heures (stade 2 blastomères), le 2^{e} globule polaire étant libéré 1 heure 15 après la ponte.

Les jeunes larves éclosent à 264 heures (±24) et le stade deux doigts est réalisé à 20 jours (±2) (Stade 39 de la table de Gallien & Durocher, 1957). C'est à ce moment que les animaux commencent à se nourrir. C'est aussi à ce stade caractéristique que sont prélevées les extrémités caudales. Il est important, comme nous le verrons, de convenir d'un horaire précis pour cette opération afin d'avoir des comparaisons valables.

Le choc thermique des œufs entraîne toujours une mortalité élevée au cours de l'embryogenèse et pendant les jeunes stades larvaires. On ne peut, sans artifice, savoir quelle est la constitution chromosomique de ces germes et en particulier il n'est alors pas possible de prélever des extrémités caudales. Pour lever cette difficulté, au moins en partie, nous avons réalisé des séries expérimentales dans lesquelles les embryons traités, survivant jusqu'au stade du bourgeon caudal, sont mis en parabiose avec un embryon normal. Ce dernier permet une longue survie d'individus qui autrement mouraient prématurément et par conséquent n'étaient pas utilisables. La méthode est particulièrement heureuse pour élever les haploïdes, certains aneuploïdes ainsi que des germes présentant de fortes hypomorphoses (Gallien, 1957). La coloration des extrémités caudales a été faite selon deux techniques: Feulgen pour les noyaux, et imprégnations argentiques pour obtenir les contours cellulaires. La technique d'imprégnation utilisée est celle de Ranvier modifiée par Chatton & Lwoff (1930): fixation au liquide de Champy, séjour dans le Da Fano salé, puis collage et enrobage dans la gélatine salée de la pièce sur lame. Celle-ci est ensuite traitée par le nitrate d'argent à 3 pour cent, à la température de +3° C. pendant 10 minutes, puis l'argent est réduit par exposition sous une lampe à U.V. pendant 5 minutes, toujours à basse température. Enfin on déshydrate et on achève le montage. La méthode de l'imprégnation argentique dessinant le contour des cellules épithéliales du tégument, il est possible non seulement d'apprécier l'hétéroploïdie par des mesures planimétriques, mais encore d'observer la structure de la nappe des cellules tégumentaires de la queue, avec les organes de la ligne latérale. Par ailleurs la régénération des queues permet éventuellement, dans un second temps, d'utiliser les méthodes de coloration nucléaire et de compter en particulier les chromosomes dans les plaques métaphasiques des cellules en mitose.

Détection et appréciation de l'hétéroploïdie après imprégnation argentique

La structure de la queue des jeunes larves est extrêmement délicate (Pl. 2, fig. 6 et 12). Au-dessus et au-dessous d'une région axiale renflée correspondant à la chorde, surmontée du névraxe et flanquée des muscles, s'élèvent les parties dorsales et ventrales de la nageoire impaire, qui s'effilent progressivement vers la bordure distale. A un niveau dorsal légèrement supérieur à celui de la moelle, on observe la chaîne des organes de la ligne latérale formant de petits renflements tégumentaires. L'axe de la nageoire est constitué par un mésenchyme très lâche dans lequel on trouve quelques corps cellulaires des cellules du mésenchyme, au nombre de 6 à 10 par coupe. Le tégument est extrêmement mince, il mesure de 10 à 15 μ d'épaisseur. Au stade considéré il comporte à la base de la nageoire deux couches cellulaires qui se réduisent à une couche dans les régions marginales. L'épithélium est de type pavimenteux. Les noyaux sont fortement aplatis. A la base du tégument des mélanophores sont visibles. L'ensemble se présente comme une lame délicate dont on peut étudier l'ensemble structural, lorsque la queue colorée et montée *in toto* est vue par sa surface. Lorsqu'on a pratiqué une coloration uniquement nucléaire, on observe la nappe des noyaux étalés dont certains sont en mitose. Après imprégnation argentique les limites cellulaires sont nettement définies. Leur aspect polygonal rappelle celui d'un parenchyme végétal (Pl. 1, fig. 2; Pl. 2, fig. 7). Pour une mise au point adéquate on peut observer les limites cellulaires dans les deux couches épithéliales et leur superposition (Pl. 2, fig. 7; Pl. 3, fig. 17).

L'épaisseur des cellules varie selon les régions. Elles sont plus hautes dans la région axiale, leur surface est plus faible que vers les bords où elles s'étalent largement. Au bord même de la nageoire, les cellules s'étirent en longueur et s'imbriquent de façon à constituer la bordure limitant la nageoire.

Aux stades de la jeune larve, précédant et suivant l'éclosion (st. 33 à 40 de la table de développement — Gallien & Durocher, 1957), la multiplication cellulaire ne compense pas exactement l'accroissement de la taille globale de la nageoire, de sorte que c'est par une extension de la surface cellulaire, surtout sur les bords, que l'accroissement de la surface de la nageoire est réalisé. De plus des mesures précises ont montré que les cellules des nappes épithéliales situées antérieurement sont relativement plus étalées que celles des nappes postérieures observées vers la pointe de l'organe (Gallien & Lambinet, 1953).

Le rapport de constance nucléocytoplasmique ($N/P = K$) permet la mise en évidence de l'hétéroploïdie par l'observation de l'ectoderme de la nageoire caudale après imprégnation argentique. En fait ce que l'on peut mesurer par planimétrie dans cet épithélium pavimenteux extrêmement aplati ce n'est pas le volume cellulaire, mais la surface des cellules étalées et dont le contour est dessiné par l'imprégnation argentique. Un examen d'ensemble permet de constater que la surface des cellules varie selon la nature de leur constitution caryologique (Pl. 1, fig. 1–3; Pl. 2, fig. 5, 7, 11). Pour tenter de préciser cette

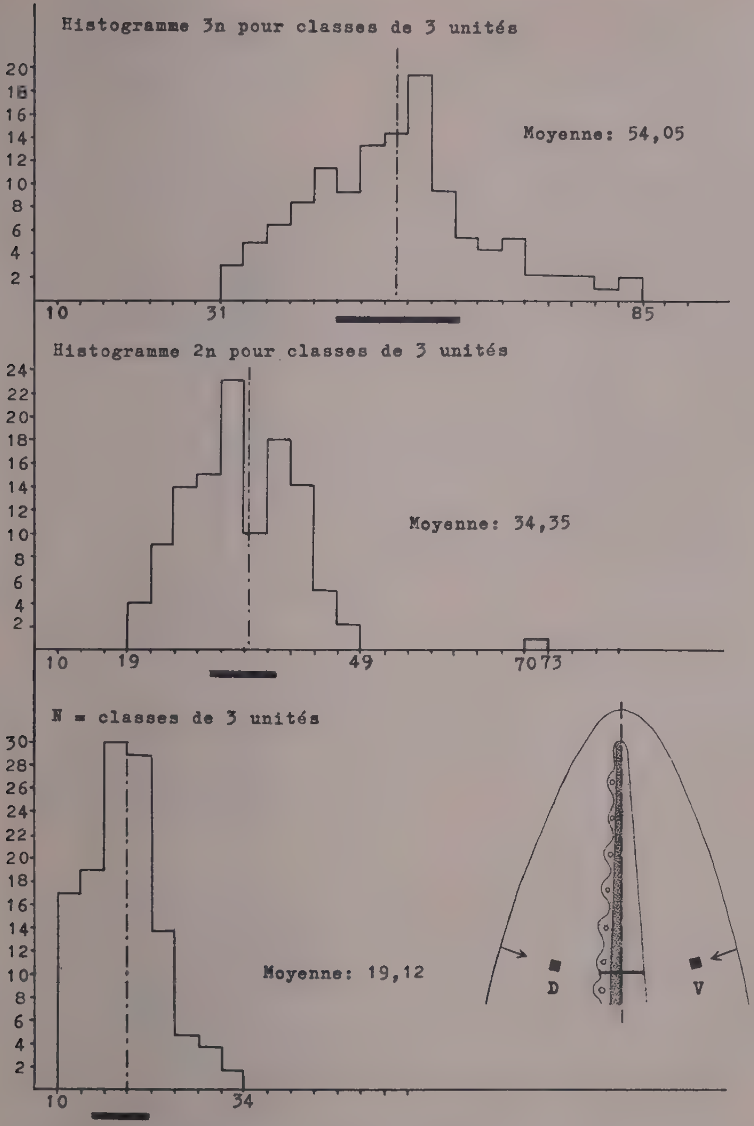

FIG. 1. Diagrammes exprimant pour l'haploïdie, diploïdie, triploïdie la variation des surfaces cellulaires exprimées par leur index planimétrique. La droite en trait-point correspond à la valeur cellulaire moyenne de l'index pour l'ensemble des 120 cellules. Sous chaque ligne d'abscisse des trois graphiques, le trait épais correspond aux valeurs cellulaires moyennes des dix individus du type considéré. Les limites du segment correspondent aux valeurs extrêmes observées (comparer avec le tableau 1). A droite, schéma d'une extrémité caudale indiquant la localisation des aires cellulaires mesurées.

observation et d'en donner une valeur chiffrée nous avons procédé de la manière suivante.

Trois séries comprenant chacune 10 larves ont été constituées pour la présente étude. Chaque lot est respectivement haploïde, diploïde, triploïde. Pour les 30 individus, et au stade 39 (table de développement de Gallien & Durocher, 1957), les extrémités caudales ont été prélevées et traitées par imprégnation argentique. Après quelques jours lorsque la queue a régénéré, on prélève à nouveau l'extrémité caudale. Les pièces sont alors colorées au Feulgen et la constitution chromosomique est établie par observation directe des plaques métaphasiques favorables (Pl. 2, fig. 8–10). Chaque lot de 10 animaux a été évidemment constitué après l'établissement du degré de ploïdie. Celui-ci étant fixé avec certitude, permet une comparaison valable avec les figures fournies par l'imprégnation argentique.

L'expression de l'hétéroploïdie a été recherchée par des mesures planimétriques. Afin de limiter au maximum les causes de variation, nous avons convenu de comparer des mesures prises sur des animaux de même stade évolutif (stade 39) correspondant au moment où débute la prise des aliments. La température d'élevage était constante et fixée à 18° C. Le choix des régions mesurées est important. Nous avons convenu de retenir deux aires, l'une ventrale, l'autre dorsale, de chacune 6 cellules. Les cellules sont mesurées individuellement. Chaque îlot est choisi selon le schéma de la figure 1. On considère de part et d'autre de l'axe correspondant à la chorde les zones D et V définies par le milieu de la distance comprise entre le bord de la nageoire et l'axe chordal et situées à une distance constante de l'extrémité caudale (1,5 mm.). Nous apprécions pour chaque animal la surface de deux aires de six cellules jointives. Pour les 10 larves de chaque lot, c'est un total de 120 cellules qui est mesuré. Les nombres fournis par les mesures planimétriques n'ont pas été convertis en surfaces réelles. Nous comparons donc des unités arbitraires.

La méthode a permis d'établir pour les trois lots respectivement haploïde, diploïde, triploïde des histogrammes traduisant les résultats des mesures (fig. 1). En abscisses sont représentées les valeurs des indices planimétriques exprimés par classes de trois unités et en ordonnées les fréquences. Ainsi que le montrent les histogrammes les valeurs individuelles des populations cellulaires chevauchent pour les trois types (*n*, 2*n*, 3*n*) considérés. Les variations observées tiennent à deux causes principales. D'une part l'épithélium n'est pas parfaitement plat, d'autre part lorsqu'on considère une aire donnée, celle-ci peut comporter deux cellules résultant d'une mitose qui vient de s'achever, ou au contraire une cellule au début d'une mitose. Dans le premier cas les cellules sont petites, dans le second la cellule est particulièrement grande. C'était le cas pour une des cellules d'un individu diploïde d'indice 70/73.

Nous devons remarquer que la variation individuelle cellulaire dans les populations triploïdes est particulièrement étendue, par rapport aux diploïdes et haploïdes. Il y a à cette situation deux raisons. Comme on peut le voir sur les photographies (Pl. 1, fig. 3), lorsque chez un individu 3*n*, on s'écarte latéralement des points D et V définis ci-dessus, les cellules épithéliales étant relativement peu nombreuses, s'étalent considérablement en direction marginale. Il est plus difficile, toutes choses égales d'ailleurs, de trouver autour des points D et V une aire de cellules homogènes, dans un individu 3*n* que dans un individu 2*n* ou *n*. En second lieu on observe parfois chez les individus identifiables comme triploïdes certaines anomalies chromosomiques sporadiques comportant l'élimination de chromosomes. Il est certain que chez de tels individus, toutes les cellules ne sont pas triploïdes (Pl. 3, fig. 14).

TABLEAU 1

Valeur de l'indice planimétrique pour 12 cellules et valeur moyenne de l'indice

Haploïdie				*Diploïdie*				*Triploïdie*			
Nombre de larves	*Surface de 12 cellules*	*Moyenne cellulaire*	*Moyenne générale*	*Nombre de larves*	*Surface de 12 cellules*	*Moyenne cellulaire*	*Moyenne générale*	*Nombre de larves*	*Surface de 12 cellules*	*Moyenne cellulaire*	*Moyenne générale*
1	182	15,1	19,12	1	363	30,2	34,35	1	552	46	54,05
2	197	16,4		2	385	32		2	606	50,5	
3	207	17,2		3	390	32,5		3	613	51	
4	213	17,7		4	391	32,5		4	618	51,5	
5	232	19,3		5	396	33		5	629	52,4	
6	242	20,1		6	400	33,3		6	632	52,6	
7	250	20,8		7	428	35,6		7	662	55,1	
8	251	20,9		8	454	37,8		8	688	57,3	
9	257	21,4		9	455	37,8		9	689	57,4	
10	268	22,3		10	466	38,8		10	746	62,1	

TABLEAU 2

Analyse des données numériques fournies par les mesures planimétriques

	Haploïdie	*Diploïdie*	*Triploïdie*
Valeurs moyennes pour 12 cellules . .	229,9	412,8	643,5
Variance . . .	737,3	1.123,4	2.660
Écart-type . . .	27,2	33,6	47,6
Coefficient de variation	11,85	12,3	14,9

Pour réduire la variabilité existant entre les types cellulaires d'un même individu, nous avons considéré pour chaque animal la mesure de la surface représentée par un ensemble de douze cellules ainsi qu'il a été indiqué (tableau 1 et fig. 1).

On constate immédiatement qu'il n'existe aucun chevauchement entre les trois groupes de mesures. L'analyse des données numériques est indiquée dans le tableau 2.

Nous avons recherché les intervalles de confiance pour chacun des trois groupes avec une sécurité de 95 pour cent. Chaque échantillon étant constitué seulement de 10 mesures il convient de porter de part et d'autre de la moyenne, 2,26 fois l'écart-type. Les résultats sont les suivants:

Haploïdie: intervalle 168 / 291,5
Diploïdie: intervalle 336,5 / 489
Triploïdie: intervalle 535 / 751,5

Ces intervalles ne chevauchent pas. Il y a donc une sécurité satisfaisante pour classer un animal dans une des trois classes d'après la mesure d'une plage de 12 cellules. Ceci justifie la méthode indirecte basée sur l'imprégnation argentique.

Finalement si nous considérons les trois séries de mesures et si nous divisons les valeurs exprimant les surfaces de chaque ensemble par les chiffres 1, 2, 3 correspondant au degré de ploïdie (1*n*, 2*n*, 3*n*), nous obtenons trois groupes de données numériques ayant sensiblement les mêmes valeurs (tableau 3). Il n'est alors pas possible de séparer statistiquement ces trois groupes. Ceci permet d'admettre que l'appréciation du contour cellulaire dans un épiderme d'épaisseur à peu près constante est bien fonction directe du degré de ploïdie.

TABLEAU 3

Données numériques obtenues en ramenant et comparant à l'haploïdie les valeurs des indices planimétriques pour la diploïdie et la triploïdie

Haploïdie	*Diploïdie*	*Triploïdie*
182	181,5	184
197	192,5	202
207	195,5	204
213	195	206
232	198	210
242	200	211
250	214	221
251	227	229
257	227,5	229
268	233	249

En conclusion, on peut dire que la méthode de l'imprégnation argentique grâce à laquelle les contours cellulaires de la nappe des cellules ectodermiques de la queue sont dessinés, permet dans les conditions définies d'apprécier le degré de ploïdie d'un individu.

Structure et évolution des nappes ectodermiques chez les aneuploïdes

La méthode de l'imprégnation argentique est particulièrement utile pour analyser la structure des nappes ectodermiques chez les individus aneuploïdes. Chez ceux-ci on observe à des degrés divers un mélange de types cellulaires

(Pl. 1, fig. 4; Pl. 2, fig. 13) dont les constitutions caryologiques sont extrêmement variables. L'observation directe des plaques métaphasiques et le comptage des chromosomes permettent de constater l'aneuploïdie pour les noyaux en mitose dont l'examen est favorable, c'est à dire pour un nombre très limité de ceux-ci. Lorsque des individus aneuploïdes sont traités par l'imprégnation argentique c'est l'ensemble des cellules et leur association qui apparaît. On peut distinguer à cet égard plusieurs catégories d'animaux.

(*a*) Chimères généralisées. Dans toute l'extrémité caudale le mélange des cellules aneuploïdes est la règle et il est très difficile de définir des types caryologiques présentant une certaine constance (Pl. 2, fig. 13; Pl. 3, fig. 16).

TABLEAU 4

Variations de l'index planimétrique exprimant les surfaces cellulaires dans un individu triploïde, présentant des îlots aneuploïdes

Aire de type généralisé triploïde (A)	*Aires aberrantes* (*B*, *C*, *D*) *aneuploïdes*				
A	*B*		*C*		*D*
70	90	36	83	29	191
65	65	35	74	28	45
55	55	34	70	23	45
51	55	33	65	23	37
48	46	31	54	22	36
47	45	25	53	21	32
44	43	22	50	12	31
42	40	7	50	9	31
		4	39		29
		2,4	34		26
			31		26
			30		25
Valeur moyenne de l'index: 55,2					24
					22

(*b*) Chimères à types cellulaires relativement localisés. Il s'agit de chimères, mais dont les cellules d'un type donné dominent dans une aire particulière par rapport aux cellules d'un autre type. Ce cas est présenté dans la Pl. 1, fig. 4. Le lobe dorsal de la nageoire est constitué d'éléments à majorité de type triploïde, cependant que le lobe ventral est formé pour sa plus grande part de cellules haploïdes. En fait ainsi qu'il est facile de le voir sur la photographie, chacun de ces groupes n'est pas homogène. Il faut parler de dominance des types triploïdes ou haploïdes, mais au sein de chaque nappe on trouve des cellules aberrantes.

La mosaïque peut intéresser non plus des aires cellulaires d'une même couche, mais les deux plans des cellules épithéliales constituant l'ectoderme dans une même aire (Pl. 3, fig. 17). Nous avons dans la zone considérée une nappe cellulaire superficielle de type triploïde et une nappe profonde sous-jacente à la précédente à dominance haploïde.

(*c*) Chimères isolées. Le cas est assez fréquent. Sur un fond cellulaire de type général haploïde ou triploïde on observe quelques îlots aneuploïdes. Ceux-ci peuvent se manifester par un ou plusieurs groupes de cellules géantes entourées de cellules *n* ou 3*n*. Souvent les aspects sont plus complexes. On observe quelques microcellules sporadiques, dont la taille est d'ailleurs variable, au sein de cellules beaucoup plus grandes. Le tableau 4 correspond aux mesures effectuées pour trois îlots aneuploïdes (B, C, D) présents dans une queue où l'ensemble est triploïde (A).

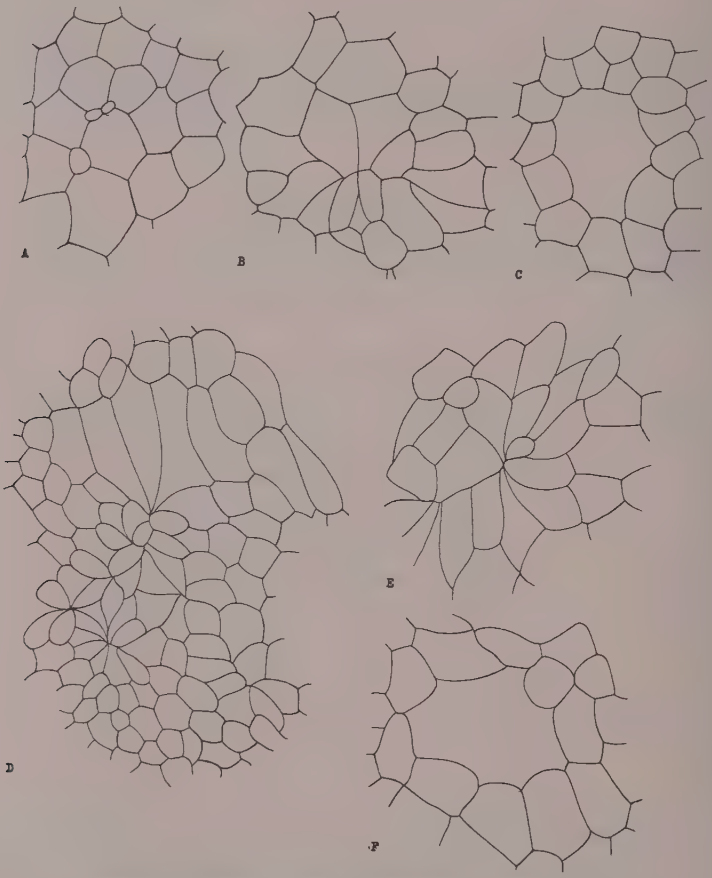

FIG. 2. Chimères localisées. Limites cellulaires et structure de la nappe épithéliale caudale, dans les aires aneuploïdes chez deux larves, respectivement *A*, *B*, *C* et *D*, *E*, *F*. En *A*, trois microcellules d'indice planimétrique 2,4; 4; 7. En *C*, cellule géante d'indice 191. En *F*, cellule géante d'indice 260. Remarquer les aspects en rosette présentés en *B*, *E*, *D*.

Les index mesurés dans une zone de type cellulaire homogène donnent une valeur moyenne de 55,2 correspondant à la triploïdie. Dans les aires aneuploïdes les index présentent des variations considérables: 2,4; 4; 5; 7; 9 pour les plus faibles, 191 pour le plus élevé. Dans un autre individu aneuploïde des cellules d'index planimétrique 211 et 260 respectivement ont été trouvées.

On observe enfin chez les individus présentant des foyers aneuploïdes plus ou moins étendus des remaniements localisés de la nappe épithéliale ectodermique (fig. 2). Dans les aires aneuploïdes on trouve des foyers autour desquels les cellules dessinent des rosettes. Ces figures représentent des zones cicatricielles. Les microcellules ou les macrocellules très fortement aneuploïdes ne sont pas viables et disparaissent. Comme elles sont localisées, les cellules voisines s'étirent pour venir combler le foyer de nécrose, aboutissant à ces aspects en rosette très particuliers. Les valeurs des index planimétriques sont dans ces zones sans signification réelle étant donné les étirements localisés qui se manifestent.

Ainsi se produisent chez les aneuploïdes des foyers d'épuration des types cellulaires les plus aberrants. Ces foyers sont autant de petites ulcérations locales qui sont vraisemblablement une cause accessoire de la mort des individus aneuploïdes, la cause principale demeurant les structures chromosomiques aberrantes.

Si dans une série importante d'œufs, de l'ordre d'une centaine, provenant d'une même femelle traités par un choc thermique on divise en deux lots les individus qui se développent, on peut considérer un premier lot coloré par le Feulgen, et un autre sur lequel on pratique l'imprégnation argentique. Il est intéressant alors de comparer les anomalies caryologiques observables dans le premier lot et les structures des nappes cellulaires du second. Dans la fig. 14, Pl. 3, il s'agit d'un individu triploïde présentant localement quelques mitoses anormales. On voit en particulier l'élimination de part et d'autre de la plaque métaphasique d'un et deux chromosomes. Près de cette figure mitotique, s'observe une série de granulations chromatiques pycnotiques. Sur un autre individu (Pl. 3, fig. 15), un micronoyau voisine avec un noyau normal. Si on rapproche ces observations des images fournies par l'imprégnation argentique, il apparaît que les micronoyaux à l'état quiescent doivent correspondre aux petits groupes de chromosomes qui dans les mitoses atypiques échappent au fuseau. Ces micronoyaux sont ceux des microcellules organisées selon le principe de constance du rapport N/P. Ce sont ces micronoyaux qui en dégénérant fournissent les granulations pycnotiques. La nécrose d'une ou plusieurs microcellules est le foyer d'une épuration cellulaire compensée par les remaniements des cellules ectodermiques voisines, venant constituer les rosettes signalées. Il est vraisemblable que les cellules géantes aneuploïdes doivent disparaître par un processus du même ordre, mais le phénomène est moins facile à caractériser que dans la formation de micronoyaux reconstitués à partir d'un petit nombre de chromosomes éliminés au cours d'une mitose atypique.

Effets tératogènes des chocs thermiques. Développement des larves hypomorphes mises en parabiose

Au cours des recherches il est apparu fréquemment à côté d'individus normaux, des larves hypomorphes à des degrés divers, mais viables: raccourcissement du tronc, anomalies du squelette des extrémités des membres (syndactylie, brachydactylie). En ce qui concerne ces dernières on peut en pratiquant l'ablation du membre et en observant la patte après régénération constater que les malformations ne sont pas en relation avec la constitution héréditaire de l'individu. Les pattes qui régénèrent à partir d'un membre atypique sont normalement constituées (Pl. 3, fig. 24). Chez les haploïdes, il arrive que l'extrémité de la chorde s'allongeant plus vite que la nageoire caudale perfore celle-ci et se coude plus ou moins en un moignon.

Dans un cas, un embryon secondaire partiel s'est développé en position ventrale sur une larve provenant d'un œuf choqué (Pl. 3, fig. 20 et 21). Au début de l'évolution de ce germe un appendice caudiforme a commencé à croître, après trois mois il se trouvait résorbé cependant qu'une paire de membres antérieurs s'était développée. Il s'agit dans cet exemple d'une altération du mécanisme inducteur. Des faits du même ordre mais consécutifs à l'hypermaturité ont été signalés par Witschi (1922, 1952) et Briggs (1941) chez les Anoures, et par Beetschen (1957) chez le Pleurodèle.

Les germes sévèrement hypomorphes (microcéphalie, microphtalmie, *spina bifida*) ou à constitution chromosomique trop aberrante ne survivent pas longtemps et en règle générale meurent au cours des stades du bourgeon caudal précédant l'éclosion. Il est possible cependant d'obtenir le développement de certains de ces germes en les mettant en parabiose avec un porteur sain diploïde. La figure 19 de Planche 3 montre l'évolution d'un germe présentant une *spina bifida*. Par cette méthode des parabioses, il est en particulier possible d'élever des individus haploïdes (Pl. 3, fig. 18). Certains ont actuellement dépassé une année.

L'état polyploïde consécutif au choc thermique de l'œuf se traduit sur le développement des organes génitaux et la physiologie sexuelle.

D'une manière générale chez les Urodèles les individus triploïdes sont stériles. Cependant chez *Pleurodeles* la stérilité est seulement partielle. Chez les femelles notamment les ovaires sont réduits à des degrés divers et les pontes comportent un faible nombre d'œufs par rapport aux témoins (Pl. 3, fig. 22 et 23). Nous avons pu obtenir un petit nombre de mâles et femelles triploïdes adultes féconds. C'est une situation comparable à celle observée par Humphrey & Fankhauser (1946, 1949) chez *Ambystoma*. Ainsi que nous l'avons mentionné cette question fera l'objet d'un travail ultérieur.

RÉSUMÉ

1. Les conditions expérimentales et d'élevage permettant d'obtenir l'hétéroploïdie chez *Pleurodeles waltlii* sont décrites.

2. Une méthode basée sur l'imprégnation argentique de l'extrémité caudale des jeunes larves permet l'appréciation de l'haploïdie, diploïdie, triploïdie. Cette méthode qui définit les contours cellulaires rend possible la mesure planimétrique d'une aire donnée de la nappe épithéliale du tégument caudal.

3. L'organisation générale de la structure des nappes ectodermiques pour l'ensemble du tégument de la queue est décrite, les altérations et régulations consécutives aux constitutions aneuploïdes sont analysées. Dans les chimères aneuploïdes, il se constitue à la suite des anomalies mitotiques des micro- et des macrocellules correspondant respectivement aux groupes chromosomiques éliminés du fuseau mitotique et aux cellules hautement polyploïdes. La disparition des éléments cellulaires les plus atypiques est suivie de mouvements et remaniements cellulaires aboutissant à des aspects en rosette, caractéristiques.

4. Quelques effets tératogènes du choc thermique de l'œuf compatibles avec la vie des animaux sont décrits.

5. Les larves fortement hypomorphes ou aneuploïdes et les individus haploïdes qui normalement ne survivent pas longtemps ont pu être élevés grâce à la pratique des greffes en parabiose, avec un individu normal.

6. Parmi les triploïdes ♂ et ♀, des individus fertiles ont été obtenus.

SUMMARY

1. Experimental conditions and methods of rearing which make it possible to obtain heteroploidy in *Pleurodeles waltlii* are described.

2. A method based on silver impregnation of the tail of young larvae permits the diagnosis of haploidy, diploidy, or triploidy. This method, by outlining the cell borders, makes possible planimetric measurement in any area of the epithelial sheet of the tail skin.

3. The general structural organization of the ectodermal layers is described for the whole of the tail skin, and the alterations and adjustments resulting from an aneuploid make-up are analysed. In aneuploid chimaeras, micro- and macrocells are formed as a result of mitotic anomalies, corresponding respectively to groups of chromosomes lost from the mitotic spindle and to highly polyploid cells. The disappearance of the most atypical cells is followed by movements and reorientations of neighbouring cells producing characteristic rosette formations.

4. Various teratogenic effects of thermal shock to the eggs, compatible with life, are described.

5. Strongly hypomorphic or aneuploid larvae and haploid individuals, which usually have a brief survival, may be reared by means of parabiotic grafting to a normal individual.

6. Amongst triploid males and females, fertile individuals have been obtained.

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EXPLICATION DES PLANCHES

Planche 1

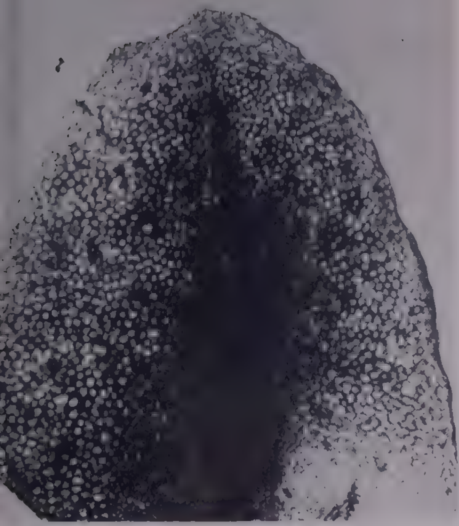
Imprégnations de l'extrémité de la queue chez la larve de *Pleurodeles waltlii*.

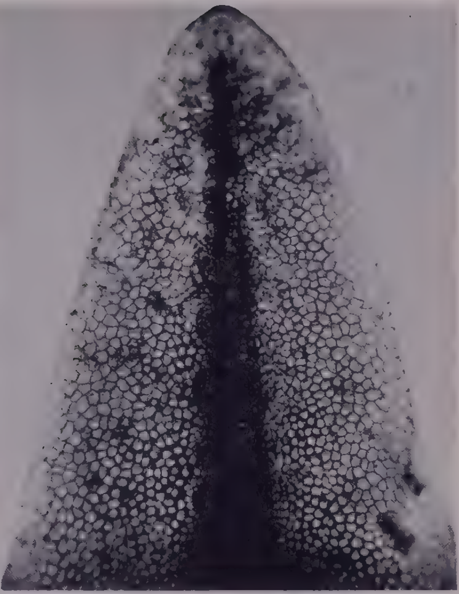
Fig. 1. Haploïdie.
Fig. 2. Diploïdie.
Fig. 3. Triploïdie.
Fig. 4. Aneuploïdie.

Planche 2

Vue à un plus fort grossissement (grossissement fig. 5) d'aires, haploïde (fig. 5), diploïde (fig. 7), triploïde (fig. 11), aneuploïde (fig. 13).

Fig. 6. Coupe transversale dans la queue d'une larve.





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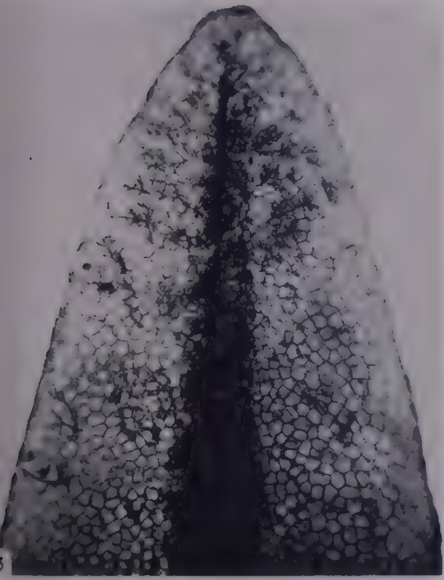

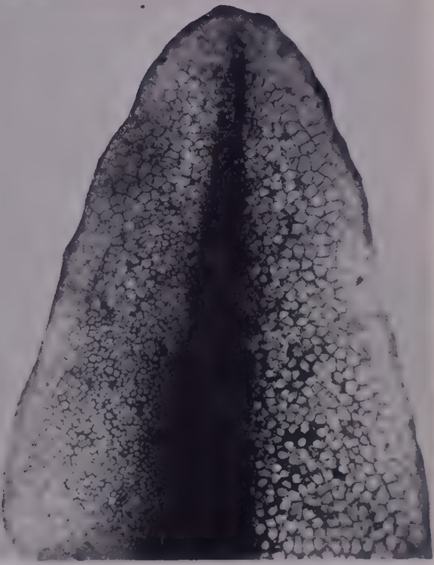

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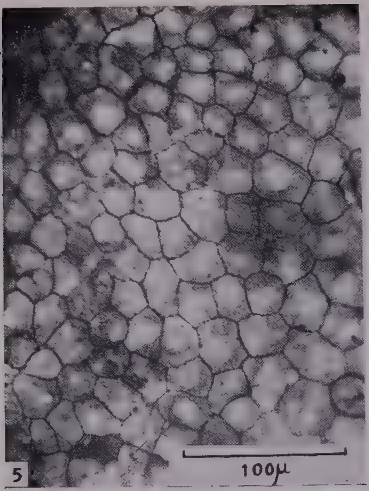
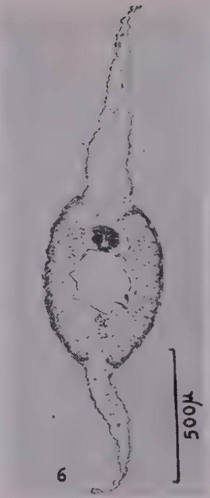
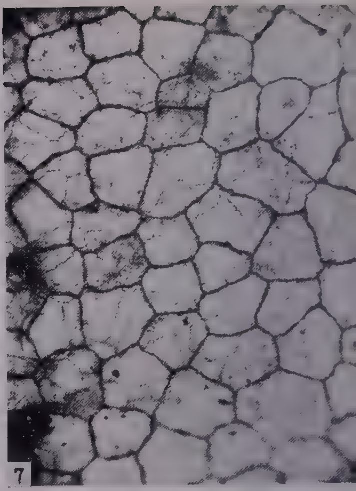
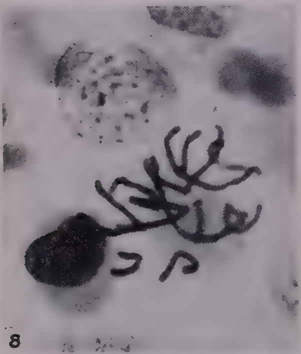
L. GALLIEN

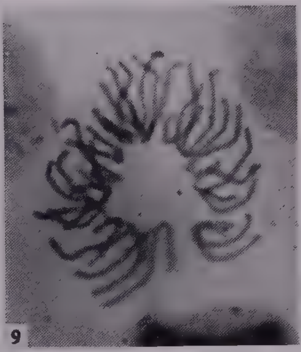
Plate 1

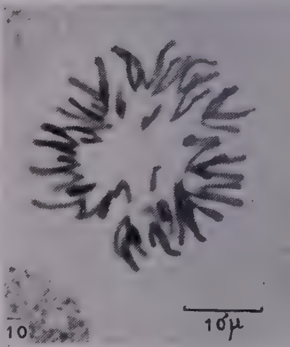


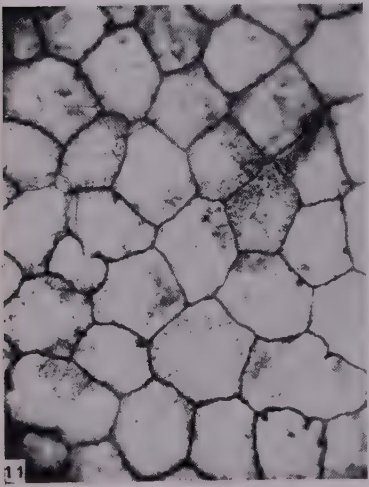


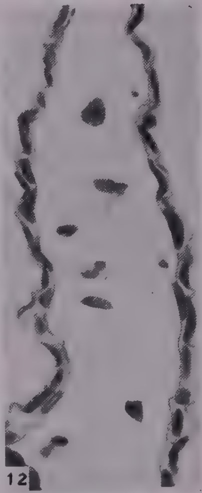












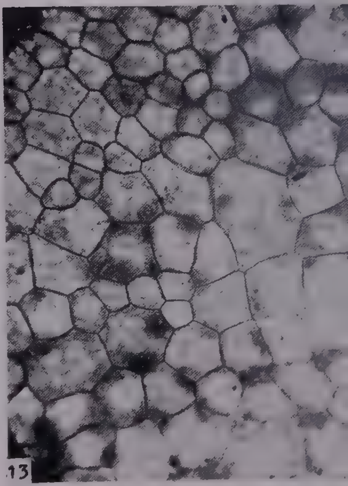


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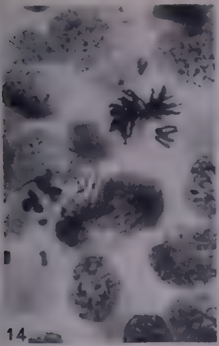
Plate 2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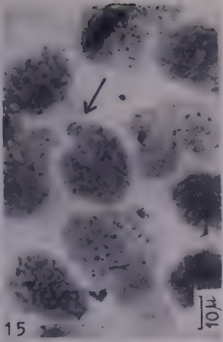
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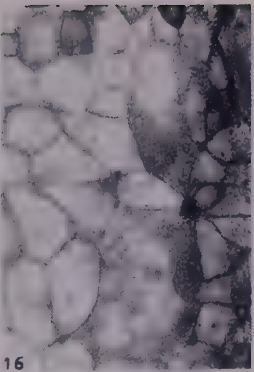
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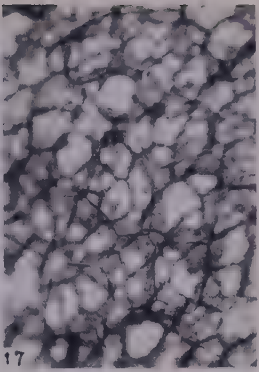
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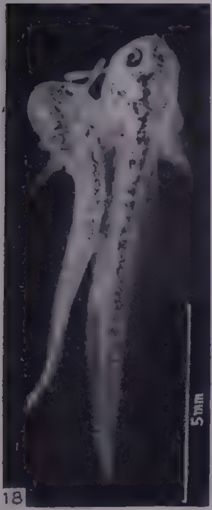
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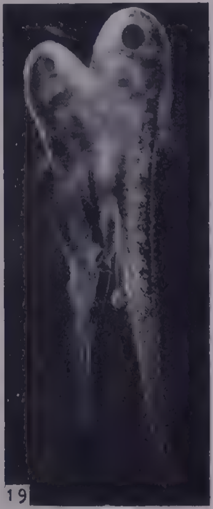
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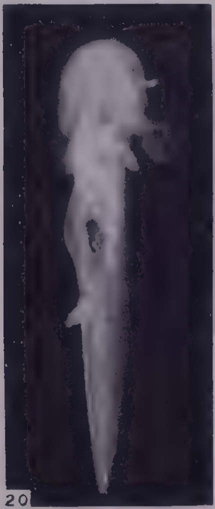
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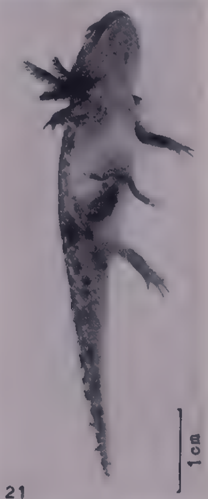
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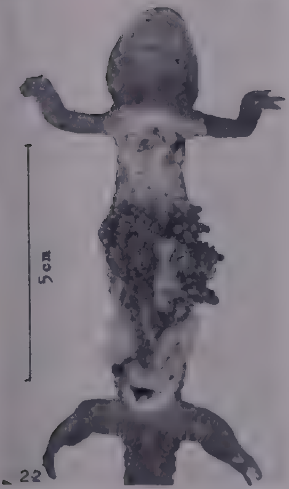
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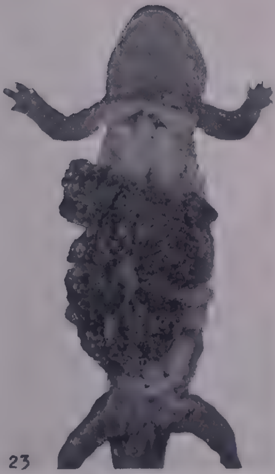
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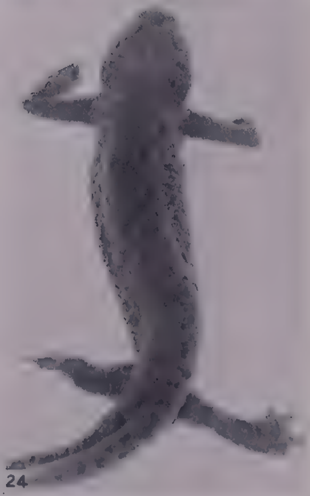
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Plate 3

Fig. 8, 9, 10. Métaphases dans les cellules de l'ectoderme caudal chez des individus respectivement haploïde, diploïde, triploïde (grossissement fig. 10).

Fig. 12. Vue en coupe du lobe dorsal de la nageoire caudale montrant l'organisation de l'épithélium pavimenteux.

PLANCHE 3

Fig. 14. Mitose atypique dans l'épithélium caudal d'un animal triploïde. Vue de profil d'une métaphase avec élimination de part et d'autre de la plaque équatoriale d'un et de deux chromosomes. En haut, masses chromatiques en pycnose (Feulgen).

Fig. 15. Micronucleus (flèche) au voisinage d'un noyau chez un animal triploïde (Feulgen). (Grossissement fig. 14–15.)

Fig. 16. Aneuploïde montrant après imprégnation argentique deux cellules géantes et l'aspect en rosette constitué par des cellules qui viennent cicatriser un point de nécrose. (Même grossissement que fig. 5.)

Fig. 17. Vue du double réseau des deux nappes ectodermiques après imprégnation argentique dans une région aneuploïde. La couche profonde est de type haploïde, la couche superficielle est triploïde. (Même grossissement que fig. 5.)

Fig. 18. Individu haploïde microcéphale (à gauche), en parabiose avec un porteur diploïde normal.

Fig. 19. Individu de 20 jours présentant une *spina bifida*, en parabiose avec un porteur normal.

Fig. 20 et 21. Deux aspects d'un individu montrant un embryon ventral incomplet, à la suite d'un choc thermique de l'œuf. Etat à l'âge de 15 jours (fig. 20). Etat à l'âge de trois mois (fig. 21). (Le grossissement des fig. 18, 19, 20 indiqué fig. 18).

Fig. 22. Ovaires hypodéveloppés d'un individu triploïde adulte (comparer avec fig. 23).

Fig. 23. Ovaires normaux d'une femelle témoin.

Fig. 24. Anomalies des membres chez un triploïde. Les quatre membres présentaient de la syndactylie. Le membre postérieur droit qui était semblable à la patte syndactyle de gauche a été sectionné. Après régénération la patte est sensiblement normale. (Grossissement des fig. 22, 23, 24 indiqué fig. 22.)

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Identification of the Type of Blood-cell Responsible for the Graft-*versus*-Host Reaction in Chicks

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INTRODUCTION

THE injection or transplantation of certain adult chicken cells into chicken embryos is known to cause a gross enlargement of the spleen and may often have fatal consequences. The enlargement produced by transplantation of adult spleen cells on to the chorioallantois has been studied by Danchakoff (1916) and by Ebert (1951, 1954). The same effect is produced by the intravenous injection of circulating white blood-cells from adult chickens (Simonsen, 1957; Terasaki, Cannon, & Longmire, 1959). The evidence of Billingham & Brent (1957, 1959), who have studied a similar phenomenon ('runt disease') in mice, and of Cock & Simonsen (1958) in chicks, indicates that these effects are due to the grafted adult cells reacting immunologically against the antigens of the helpless host. This type of reaction has been called the 'graft-*versus*-host' or graft against host reaction.

If this explanation of the mechanism of splenic enlargement is accepted, it seems that it might be possible to obtain pure types of white cells to determine which kind can cause the reaction. Thus it might be possible to identify the cell responsible for not only the graft against host reaction, but also, perhaps, for the homograft reaction itself. This task has been simplified by Simonsen's (1957) demonstration that it is possible to propagate the agent responsible for splenic enlargement by serial transfer, i.e. by the removal of the blood of chicks previously injected with adult blood and its injection into other embryos. The activity could be transferred through nine successive passages. This behaviour seems to eliminate the polymorphonuclear leucocyte, which is generally thought not to reproduce and, accordingly, attention has been concentrated on the lymphocyte and monocyte. Lymphocytes were obtained from the blood about 99 per cent. pure. Monocytes were obtained presumably 100 per cent. pure from tissue cultures of white blood-cells. Two main types of tests for the graft against host reaction were employed. First, the test described above, viz. weighing the spleen 5–7 days after the intravenous injection of various adult chicken cells into chick embryos; and second, the intracutaneous injection of cells into young chicks to

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test for the production of a local inflammatory swelling. Evidence will be given that the production of this skin reaction is at least partly a result of a graft-*versus*-host reaction (see also Cock & Simonsen, 1958).

METHODS

The breeds or strains of chickens used in these experiments were: Light Sussex (LS), Rhode Island Red × White Leghorn cross (R × L), and the inbred *I* and *W* line chickens. The inbred lines were developed by A. G. Cock and have been tested for homogeneity by skin grafting (Cock & Clough, 1956). Fertile eggs were supplied by the Northern Breeding Station, Reaseheath, and the author is most grateful to the Station and to the Agricultural Research Council for making them available.

Splenic enlargement tests

The general scheme of this series of tests was to prepare cells from adult chickens and inject them intravenously into embryonic chicks at 11–18 days' incubation. The recipient chicks were then sacrificed after 5 days or more, and the spleens weighed.

Injection of serially diluted adult whole blood. Citrated blood from a LS hen was diluted serially in 1:3 dilutions. Seven dilutions were prepared and groups of ten 14-day incubated R × L embryos were injected with 0·1 c.c. of each dilution. The mortality of the embryos was checked daily and the spleens of the dead embryos were weighed.

Lymphocyte preparation and injection. Lymphocytes were obtained from the blood by some modifications of a technique described by Jago (1956) for isolation of human blood lymphocytes. Jago's technique consists essentially of lightly centrifuging heparinized blood and drawing off the uppermost cells of the buffy coat (lymphocytes) with a pipette. By the use of this method it has been readily possible to obtain lymphocytes 97–98 per cent. pure, as claimed by Jago. In an attempt to obtain purer preparations the following modifications were made. Blood obtained by cardiac puncture and containing 10 units of heparin per ml. was incubated in 50-ml. centrifuge tubes for about one hour at 37° C. During this incubation period polymorphonuclear leucocytes and monocytes stick in large numbers to the glass surface of the centrifuge tube and also phagocytize red blood-cells, thus becoming heavier. Incubation in smaller centrifuge tubes is preferable in presenting a relatively greater surface area of glass, but is somewhat troublesome when handling large quantities of blood. The blood is then centrifuged very lightly at about 200 g. for 3–5 minutes. The blood-cells should be packed only lightly by this procedure, leaving about one-quarter of the total volume as plasma. This degree of packing of cells is a better index than the force of centrifugation, since the degree of sedimentation of blood-cells varies considerably between different individuals. The plasma is then drawn off with a hypodermic needle attached to a syringe. Since the force of suction with a hypodermic needle is not directly downwards, the plasma can be drawn out with a minimum of disturbance of the buffy coat. The upper layers of the buffy coat consist mainly of lymphocytes, however, and the extent to which these cells were drawn up—which depended largely on the position of the point of the needle in relation to the surface of the buffy coat—was decided by whether a higher yield or greater purity was desired. The plasma containing lymphocytes was then centrifuged at 1,000 g. for 7 minutes to concentrate the cells. To ascertain the degree of purity, the cells were examined by phase-contrast and ordinary microscopy after vitally staining with neutral red, a procedure which facilitates the identification of polymorphonuclear leucocytes and monocytes. This procedure also enables one to determine whether a given polymorphonuclear leucocyte or monocyte is dead or alive. The number of contaminating cells was expressed in all cases as a proportion of at least 1,000 counted lymphocytes.

Lymphocyte purification was also attempted by first isolating the lymphocytes by the above technique and then culturing the cells in Carrel flasks for 8 or 20 hours to allow any contaminating polymorphonuclear leucocytes and monocytes to stick to the glass surface. After incubation the supernatant fluid of the culture was taken off gently, examined, counted, and injected into embryos.

Lymphocytes from chicks previously made tolerant or immune to tissues of inbred chicks were injected in the manner described above. Tolerance (i.e. specific immunological non-reactivity) was produced by the injection of 500,000 *I* strain lymphocytes into a 16-day R × L embryo. On the 16th day after hatching, when the chick seemed sick (presumably as a consequence of the graft-*versus*-host reaction), its lymphocytes were removed for injection into *I* strain embryos. Chicks were *immunized* either by skin grafting or injection of spleen-cells intravenously and intraperitoneally. The spleen-cells were prepared by mincing the spleen with scissors, teasing out the fragments under Ringer-phosphate, letting the cell suspension settle 20–30 minutes, and taking the upper half of the suspension for intravenous injection and the lower half for intraperitoneal injection. For intravenous injection, the cells were washed once with Ringer-phosphate. In all cases the immunizing tissue was taken from an inbred donor and the host was either R × L or LS. The host lymphocytes were injected into embryos of the same inbred line as the donor chicken, i.e. of the inbred line against which the state of immunity was directed.

Lymphocyte dilution. Lymphocytes were prepared from normal adult *I* strain blood by the method described above. Since the preparation still had contaminating cells, it was incubated for a further 30 minutes and recentrifuged at 200 g. for 2 minutes. In the final suspension, no live polymorph or monocyte could be found per 1,100 lymphocytes. This suspension was diluted six times successively by one-quarter. Cells at each dilution were injected intravenously into ten 14-day embryos. Five days later all 60 embryos were sacrificed, their spleens weighed, and the mean splenic weights plotted (see Text-fig. 1).

Monocyte culture and injection. Monocytes were obtained by culturing the buffy coat of adult chicken blood for periods of 1–10 days. In ordinary cultures, monocytes were found to adhere strongly to glass surfaces and could not be removed either by trypsin or versene treatment or by a combination of both. Consequently, monocytes were grown on a chicken plasma clot which was later dissolved with trypsin, thus freeing the monocytes growing on its surface. The cells were grown in Carrel, Rous, and Thompson flasks. A 10–30 per cent. solution of chicken serum in Ringer-bicarbonate was employed as the culture medium. After culturing, the supernatant medium was removed and the plasma clot washed three times with 10–15 ml. of Ringer-phosphate to remove any cells not firmly attached to the clot. The clot was then digested for 10–20 minutes, at room temperature or 37° C., with 2–4 c.c. of 0·5 per cent. 1:250 Difco trypsin per Carrel flask. Three volumes of Ringer-phosphate were used to rinse out the flasks and dilute the trypsin. The pooled washings were centrifuged at 1,000 g. for 8 minutes. Counts of the resuspended cells were taken together with counts on neutral red preparations to correct for the number of viable monocytes. Finally, the cell suspension was injected intravenously into 11–18 day chick embryos. The splenic weights were taken of embryos which died, and of surviving chicks killed at either 1 day or 7–8 days after hatching. As a test for viability after trypsinization, the monocytes were subcultured in 3 cm. diameter Carrel flasks with an equal mixture of Ringer-phosphate and 'conditioned' culture medium, i.e. medium taken from culture flasks with growing cells. Conditioned medium by itself was also cultured as a control to establish that it did not contain viable cells.

Thymic cell injection. One lobe of the thymus was removed and was either teased with forceps under Ringer-phosphate or put through a sieve into Ringer-phosphate. After standing for 20–30 minutes the upper supernatant cells were taken out, washed once, counted and injected into 15- or 16-day embryos. The thymic cell donors were either 3, 12, or 21 weeks old. Recipient embryos were sacrificed and their spleens weighed 6 days after injection.

Injection of adult spleen cells. Cells from a LS chicken spleen were teased out gently in a medium containing 9 volumes of Ringer-phosphate and 1 volume of 1 per cent. gelatin in normal saline. The suspension was drawn gently in and out of a pipette and allowed to settle for 10 minutes, and the top layers were then taken off. From this, a suspension was obtained containing 16 million cells per ml., excluding red blood-cells. Five further serial dilutions of 1:5 were prepared for injection into 14-day R × L embryos. Each dilution was injected into groups of 12 embryos (0·5 ml. per egg). On the 5th day after injection the embryos were killed and the spleens weighed.

Production of nodules in the skin

Cells prepared in various ways were injected intracutaneously. Each site was injected with 0·05 ml. of the cell suspension with a 30-gauge needle. The injection sites were examined daily from the 1st day, or in the later experiments from the 3rd day. The intracutaneous nodules were measured with a ruler, an approximate volume calculated, and the mean volume for each group of chicks obtained. Ratings of — to 4+ were assigned as follows: 0–5 mm.^{3}, —; 5–10 mm.^{3}, +; 10–50 mm.^{3}, 2+; 50–100 mm.^{3}, 3+; 100–250 mm.^{3}, 4+.

In group numbers 1 to 15 (Table 5), 5 million lymphocytes prepared from normal adult chickens by the methods described above were injected repeatedly as indicated. Similar single and repeated injections of normal adult lymphocytes were made into chicks previously made tolerant to tissues isologous with the lymphocytes (groups 16–25). Chicks of group 16 were made tolerant by the injection of 5 million lymphocytes intravenously at the time of hatching. Chicks of group 17 were also injected intravenously with lymphocytes at hatching and, in addition, their state of tolerance was checked by the transplantation of skin homografts 30 days after grafting, at which time lymphocytes from the same donor were injected intracutaneously. Chicks of groups 18 and 19 were made tolerant by previous injection with lymphocytes when they were 16-day-old embryos, though the state of tolerance was not in this case checked by skin grafting. Thymocyte suspensions were identical with those used in the tests for splenic enlargement. Both thymus suspensions were injected in two different areas on the back of 5 chicks. Monocytes were obtained in the manner already described.

RESULTS

Splenic enlargement tests

Serial dilution of whole blood. When injected into 14-day-old embryos, adult whole blood diluted serially by one-third produced a decreasing mortality (Table 1). Splenic enlargement was found at a dilution of 1:27, each inoculum containing 122,000 lymphocytes, 73,000 polymorphonuclear leucocytes, and

TABLE 1

Mortality and splenic enlargement produced in embryos by injection of serially diluted adult whole blood

Blood taken from a LS chicken was serially diluted by one third, and 0·1 ml. of each dilution injected into 14-day R × L embryos

Dilutions of blood	*Number of embryos injected*	*Number of dead embryos or chicks by the 8th day after injection*	*Mean splenic weight of dead embryos per chicks*
			(mgm.)
1	9	6	83·0
1 : 3	10	5	60·0
1 : 9	10	4	76·3
1 : 27	9	4	46·7
1 : 81	9	4	6·5
1 : 243	10	3	6·8
1 : 729	8	2	7·9

TABLE 2

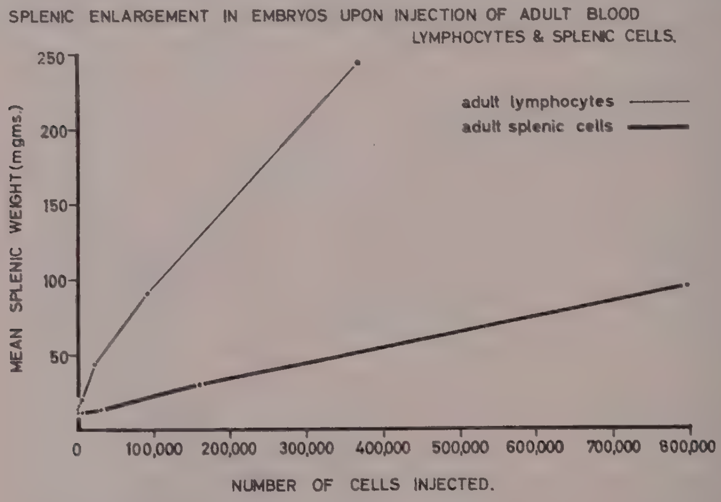
The splenic weights of chick embryos injected with adult blood lymphocyte preparations

Prep. no.	*No. of cells injected*			*Previous treatment of donor and donor cells*	*Donor breed*	*Host embryo*			
	Lymphocytes	*Polymorphs*	*Monocytes*			*Breed*	*Age*	*No. embryos injected*	*Mean splenic weight* (mgm.)
5	30,000	166	83	None	LS	R×L	14	8	38·0
4	60,000	83	83	None	LS	R×L	14	8	34·4
5	180,000	1,000	500	None	LS	R×L	14	7	76·0
6	210,000	215	430	None	R×L	*W*	17	9	32·7
4	360,000	500	500	None	LS	R×L	14	10	140
1	1,700,000	12,000	19,000	None	RIR	LS	14	5	49·2
2	1,800,000	38,000	74,000	None	RIR	LS	14	5	47·0
7	100,000	Not counted	Not counted	None	*I*	*I*	16	9	25·2
7	500,000	Not counted	Not counted	None	*I*	*I*	16	11	67·5
8	1,000,000	3,600	3,600	None	*I*	*I*	16	7	16·0
9	5,000,000	10,000	19,000	None	*I*	*I*	17	4	11·7
10	150,000	20,000	54,000	Normal donor—lymphocytes cultured 8 hours over plasma clot	LS	*W*	14	5	116
11	200,000	0	0	Normal donor—lymphocytes cultured 8 hours on glass	LS	*W*	14	5	90
11	1,000,000	0	0		LS	*W*	14	5	57
12	350,000	2,000	1,400	Normal donor—lymphocytes cultured 20 hours on glass	*W*	R×L	15	7	14·9
13	1,500,000	9,000	0	Tolerant to *I* line cells	R×L	*I*	16	5	57
14	1,000,000	23,000	47,000	Immunized by i.v. inj. of spl. 6 days prev.	LS	*W*	14	5	37·2 (5/5)*
15	1,100,000	47,000	55,000	Immunized with skin graft 6 days prev.	R×L	*W*	14	6	16·2 (3,6)*
16	1,300,000	260	1,600	Immunized by i.v. and i.p. inj. of spleen cells	*W*	*I*	16	4	21·6 (0,4)*
17	1,900,000	2,500	20,000	Immunized with skin graft 16 days prev.	R×L	*W*	15	5	38·0 (5/5)*
16	5,100,000	1,100	6,100	Immunized by i.v. and i.p. inj. of spl. cells 6 days prev.	*W*	*I*	16	5	35·1 (0/5)*
18	1,000,000	Spleen cells	. .	From same hen as lymph. prep. No. 14	LS	*W*	14	6	21·1 (5/6)*
	Controls	No cells injected				R×L LS *W*, *I*		26	16·0 (1·2)

* Numerator = number of embryos with necrotic spots in the skin; denominator = number of embryos examined.

32,000 monocytes. Even allowing for some error in counting, it can be seen that relatively small numbers of cells can produce a detectable enlargement. These preliminary figures were used to determine the number of cells which should be injected and the limits of allowable contamination.

Blood lymphocyte injection. As can be seen in Table 2, five preparations of normal adult blood lymphocytes all caused splenic enlargement. Comparisons between preparations is difficult since different strain combinations were used. It is important to note here that preparations containing 30,000–360,000 lymphocytes and 83–500 monocytes can cause splenic enlargement. If we compare these figures with the number of cells present in blood diluted to 1:27 (Table 1) it will be seen that the number of lymphocytes matches reasonably well, while the number of monocytes does not: if the 83–500 contaminating monocytes were responsible for the splenic enlargement, diluted blood should have been effective down to dilutions of about 1:1,700.



TEXT-FIG. 1. Effect of graded dosages of adult chicken splenic cells and lymphocytes on the splenic weight of chick embryos. Fourteen-day-old R × L chick embryos were injected i.v. with adult *I* strain lymphocytes and LS splenic cells. Five days after injection the embryos were killed and wet weights of the spleens were taken. Each dilution of cells was injected into 10–12 embryos.

After injection of lymphocytes from inbred *I* strain adults into *I* eggs (preparations 7, 8, 9; see Table 2), some enlargement was produced, especially with preparation 7. However, it is important to note that in this group two embryos had very large spleens weighing 215 and 165 mgm., so contributing disproportionately to the mean. Such an effect might be due to residual antigenic diversity of the *I* line (cf. Cock & Simonsen, 1958). If we exclude these two cases, the mean splenic weight of the group injected with 500,000 isologous lymphocytes becomes

40·2 mgm. This is considerably less than the 250 mgm. splenic weights produced by injection of 300,000 homologous lymphocytes (Text-fig. 1).

As another method of purifying the lymphocyte preparation, lymphocytes were cultured in the hope that monocytes and polymorphonuclear leucocytes would stick to the bottom of the flask. Lymphocytes cultured over plasma clot for 8 hours did not yield a pure preparation, but cultures on glass yielded an essentially 100 per cent. pure preparation. This suspension did produce marked enlargement. Culture for 20 hours did not yield as pure a lymphocyte preparation, and this preparation did not produce splenic enlargement, perhaps because of the low viability of lymphocytes after 20 hours of culture.

In one small experiment lymphocytes from a chick tolerant to *I* line cells injected into *I* line embryos produced splenic enlargement (prep. 13).

Lymphocytes obtained from chickens immunized to *I* or *W* line tissue produced some splenic enlargement when injected into embryos isologous with the immunizing tissue (preps. 14–17). Lymphocytes from immunized animals did not produce deaths at an earlier time than normal blood-cells, but produced macroscopically visible necrotic spots in the skin. The only variable apparently correlated with the presence or absence of necrotic spots was the strain combinations (extreme right-hand column, Table 2). When the donor and recipient strains were either LS or R × L to *W*, 1–8 necrotic spots were found. When the combination of *W* to *I* strain was tested, no spots were found.

Spleen cells from immunized chicks also did not cause marked splenic enlargement or deaths earlier than would be expected after the injection of normal spleen cells. However, spots again were found in the skin of 5 out of 6 embryos.

Lymphocyte dilution. Five dilutions of essentially 100 per cent. pure blood

TABLE 3

The splenic weights of chick embryos injected with adult chicken monocytes

No. of viable monocytes injected*	*No. of days monocytes cultured previous to injection*	*Incubation age of injected embryo*	*Spleen weighed at 19 days' incubation–1 day post-hatch*		*Spleen weighed at 7–8 days post-hatch*	
			No. of embryos injected	*Mean splenic weight*	*No. of embryos injected*	*Mean splenic weight*
				mgm.		mgm.
15,000–17,000	7	11	2	10·9	1	116
18,000–58,000	10	14	3	9·1	4	154
18,000–69,000	7	14–18	4	11·1	..	..
60,000–75,000	1	17	8	12·5	..	..
112,000–258,000	5–7	14–15	5	11·2	5	116
400,000–620,000	5–6	14–15	12	19·2	..	..
TOTAL			34	14·1±1·5	10	131±12·8
CONTROLS, no monocytes injected			26	16·0±1·2	10	98·7±18·1

* viability as judged by ability to pick up neutral red dye.

lymphocytes caused an increase in the weight of the spleen in almost direct proportion to the number of cells injected (Text-fig. 1). From the graph it can be seen that an injection of as few as 30,000 lymphocytes can cause a definite enlargement of the spleen. This figure compares reasonably well with the concentration of lymphocytes in whole blood diluted to 1:27. At this dilution of blood about 120,000 lymphocytes were found to be present. The fourfold discrepancy could be accounted for by the different breed combination used. The discrepancy between the number of monocytes in the lymphocyte preparations and the 1:27 diluted blood, on the other hand, is roughly 30,000 fold.

Monocyte injection. Blood monocytes cultured for 1–10 days and injected in dosages of 15,000 to 620,000 cells did not produce any sign of splenic enlargement (Table 3). Because cultured monocytes may require more time to readjust to *in vivo* conditions, some chicks were killed about 2 weeks after injection. These chicks had slightly but probably not significantly enlarged spleens. Since it is possible, too, that monocytes may lose their activity on prolonged culture, monocytes cultured for 1 day were also tested—with identical results. The same negative results were obtained with 1-day cultured monocytes in the skin reaction tests to be described below. That the monocytes were viable was shown by their ability to pick up neutral red dye, and to proliferate when recultured.

TABLE 4

The splenic weights of chick embryos injected with adult thymus cells

Thymic cells donor		*Recipient embryo breed*	*No. of thymic cells injected* ×10°	*No. of embryos injected*	*Mean splenic weight*
Breed	*Age*				(mgm.)
W	3 weeks	R×L	1·0	5	13·9
W	3 weeks	R×L	0·5	5	18·6
W	3 months	R×L	1·0	5	32·3
W	3 months	R×L	0·5	5	21·6
R×L	5 months	LS	1·1	3	13·1
R×L	5 months	LS	11·0	3	43·3
R×L	5 months	LS	33·0	3	30·6
CONTROLS—no cells injected				26	16·0+1·2

Thymic cell injection. Thymic cells injected into embryos produced a one- to threefold enlargement of the spleen (Table 4). This enlargement was, however, obtained by injecting large numbers (in the order of millions) of thymic cells. The amount of contamination with blood lymphocytes could not be determined because of the difficulty in distinguishing thymocytes and small lymphocytes. Previous data make it clear that contamination with lymphocytes in the order of 2 per cent. could account for the degree of splenic enlargement obtained.

Splenic cell injection. Spleen cells from an adult chicken injected into

TABLE 3

The production of nodules in the skin of chicks by injection of blood lymphocyte preparations containing five million lymphocytes from normal adult chickens

Group number	*Donor breed*	Recipient Chicken				Approximate mean volume* of nodules at:		
		Breed	*Age*	*Previous treatment*	*No. of chicks injected*	*3 days*	*4 days*	*5 days*
1	*W*	LS	12 hrs.	None	10	+	+ + +	+ + + +
2	*I*	R × L	1 day	None	5	—	+	+ + +
3	*W*	R × L	1 wk.	None	10	+ + +	+ + +	. .
4	*W*	LS	2 wks.	None	9	+	+ +	+ +
5	*W*	R × L	3 wks.	None	12	+ +	+ + +	. .
6	*I*	LS	3 wks.	None	5	+ + +	+ + + +	+ + +
7	*I*	*I*	2 days	None	4	—	—	—
8	*I*	*I*	4 days	None	5	—	—	—
9	*W*	R × L	4 wks.	2nd i.c. injection. Inj. with lymphocytes 7 days prev. (group 5)	12	-	-	—
10	*W*	LS	3 wks.	2nd i.c. injection. Inj. with lymphocytes 10 days prev. (group 4)	9	—	—	-
11	*I*	R × L	1 wk.	2nd i.c. injection. Inj. with lymphocytes 6 days prev. (group 2)	5	—	—	—
12	*W*	R × L	5 wks.	3rd injection. Inj. with lymphocytes 6 days prev. (group 9)	11	+ +	+ +	—
13	*I*	R × L	4 wks.	3rd injection. Inj. with lymphocytes 18 days prev. (group 11)	5	+ + +	+ + + +	+ + +
14	*I*	R × L	6 wks.	4th injection. Inj. with lymphocytes 10 days prev. (group 13)	4	+ +	+ +	+
15	*I*	R × L	7 wks.	5th injection. Inj. with lymphocytes 6 days prev. (group 14)	4	+ +	+ + +	+ + +
16	*W*	LS	12 hrs.	Inj. i.v. with 5 m. lymphocytes at same time as i.c. injection	5		+ +	+ + + +
17	*W*	R × L	4 wks.	Tolerant chicks with surviving homografts from lymphocyte donor	7	+ + +	+ + +	. .
18	*I*	R × L	4 wks.	Injected prev. with lymphocytes at 16 days of incubation	5	+ +	+ +	+
19	*I*	R × L	1 day	Injected prev. with lymphocytes at 16 days of incubation	4	+ +	+ + +	+ + +
20	*I*	R × L	5 wks	2nd injection. Inj. with lymphocytes 10 days prev. (group 18)	5	+ + +	+	
21	*I*	R × L	1 wk.	2nd injection. Inj. with lymphocytes 6 days prev. (group 19)	4	+ +	+ +	+ + + +
22	*I*	R × L	6 wks.	3rd injection. Inj. 6 days prev. with lymphocytes (group 20)	4	-	+ +	
23	*I*	R × L	4 wks.	3rd injection. Inj. 18 days prev. with lymphocytes (group 21)	4	+ + +	+ + + +	+ + +
24	*I*	R × L	6 wks.	4th injection. Inj. 10 days prev. with lymphocytes (group 23)	3	+ +	+ +	+ +
25	*I*	R × L	7 wks.	5th injection. Inj. 6 days prev. with lymphocytes (group 24)	3	—	-	

* Skin nodules measured with a mm. ruler, an approximate volume calculated, and the mean volume for each group obtained. Ratings of — to + + + + were assigned as follows: 0–5 mm.³ = —; 5–10 mm.³ = +; 10–50 mm.³ = + +; 50–100 mm.³ = + + +; 100–250 mm.³ = + + + +.

embryos produce marked splenomegaly in 5 days. Injection into groups of chicks of six serial 1 : 4 dilutions of the spleen-cell suspension showed that about 100,000 cells could produce a definite enlargement (Text-fig. 1). As with the lymphocyte injections, the weight of the spleen was almost directly proportional to the number of cells injected. The graph shows that the slope of the line is less than that produced by lymphocyte injections, but it is somewhat difficult to compare the two effects because of the difference in strain combinations.

Production of nodules in the skin

Normal adult lymphocytes injected into the skin of normal young chicks produced large nodules in 3–5 days (Table 5). Usually no reaction was macroscopically visible in the first 2 days after injection. The nodule appearing on the 3rd day usually reached its maximum size at 4–5 days and then regressed, in all cases, within 8 days. That this reaction is primarily a homograft reaction is supported by the observation that injection of adult *I* line lymphocytes into isologous chicks resulted, essentially, in no reaction (Table 5, groups 7 and 8).

Second injections of lymphocytes (into homologous chicks) from chickens isologous with the donor of the first lymphocytes produced virtually no reaction (groups 9–11). However, third, fourth, and fifth injection of lymphocytes again produced nodules.

In chicks previously rendered tolerant (groups 16–19) to cells of the lymphocyte donor the first intracutaneous lymphocyte injection produced nodules roughly of the same size as in normal chicks. The second injection of lymphocytes from the same donor strain, however, produced a considerable reaction, in contrast to the negative reaction obtained in normal chicks upon the second injection. The reaction became weaker on the third injection in one group (22) and negative only upon the fifth injection in another group of chicks (group 25).

In two small series of experiments (Table 6), lymphocytes from tolerant and immune chicks did not produce any marked reaction in the skin. Thymocytes from normal 3-week and 3-month-old chickens also had very little effect. Monocytes from 7-day cultures produced only a small nodule. Normal adult white blood-cells cultured on a plasma clot for 1 day were divided into two groups for injection in two separate sites on the same chicks: the floating supernatant cells and the cells attached to the plasma clot. The main difference between the cellular compositions of the two groups was that monocytes were concentrated in the clot but hardly present in the supernatant. The supernatant cells caused the formation of nodules in the skin, whereas the cells which had been attached to the plasma clot did not. It would thus seem quite definite that monocytes at least do not cause nodule formation. The possibility that trypsin treatment used to dissolve the clot had destroyed the activity of the active cells was tested by injection of trypsinized and untrypsinized lymphocytes. These two suspensions injected into the skin of the same chicks produced reactions of equal severity.

TABLE 6

Production of nodules in the skin of normal untreated chicks by various adult cells

	Donor chicken	*Recipient chicken*					
					Mean volume of nodules at:		
Breed	*Previous treatment of donor, amount and type of cell injected*	*Breed*	*Age*	*No. of chicks injected*	*3 days*	*4 days*	*5 days*
R×L	Lymphocytes from chickens made tolerant to *I* line, still retaining surv. grafts—5 m. lymphocytes	*I*	2 days	3	..	..	+
I	Lymphocytes from chicken inj. i.v. 7 days prev. with *W* spleen—4·8 m. lymphocytes	*W*	12 hrs.	4	..	+	++
W	Thymocytes from normal 3-week-old chicken—6·3 m.	*I*	5 days	5	..	..	..
W	Thymocytes from normal 3-month-old chicken—5·0 m.	Same chicks as above			..	+	..
S	Monocytes from 7-day cultures of WBC from a normal chicken—1·5 m.	*W*	12 hrs.	5	..	+	..
I	Cells from a 1-day culture of WBC from normal chickens. 3·2 m. monocytes and 3·2 lymphocytes	*LS*	2 days	5	..	..	..
I	Cells in the supernatant of the above 1-day culture of WBC. 0·09 m. monocytes 2·6 m. lymphocytes.	Same chicks as above			..	++	++
R×L	Lymphocytes divided into two aliquots 9 m. lymphocytes trypsinized in same manner as in prep. of above monocytes.	*LS*	5 wks.	4	++	+++	+
	Second aliquot, untrypsinized—9 m.	Same chicks as above			++	+++	+

DISCUSSION

The principal aim of this work was to determine which type of cells is responsible for the graft-*versus*-host reaction. If splenic enlargement and skin nodule development represent a homograft reaction in reverse, with the graft reacting against the helpless host (Billingham, 1958; Billingham & Brent, 1957, 1959; Simonsen, 1957; Cock & Simonsen, 1958), the presumption is strong that the cell which can produce such a reaction is responsible also for the ordinary type of homograft reaction. (It should not be forgotten, however, that *iso*-immune reactions other than those concerned with transplantation immunity will be set in train by grafting of adult lymphoid cells to young animals—e.g. the antibodies responsible for haemolytic anemia (Simonsen, 1957). Since, in chicks, peripheral white blood-cells can cause splenic enlargement, and since the responsible cell has been shown by Simonsen (1957) to be capable of reproduction, it remained to be shown whether monocytes or cells of the lymphoid family cause the reaction.

At this point it may be necessary to review the evidence for considering the production of skin nodules by the injection of homologous cells as a graft against host reaction. Nodule development is clearly due to a homograft reaction because lymphocytes from inbred chickens injected into chicks of the same inbred strain cause no reaction. It seems to be a result of a graft-*versus*-host reaction rather than a reaction of the host against the graft because a nodule is produced by lymphocytes upon the first injection, but not after a second injection. If the development of the first nodule was due to a host against graft reaction, then an even greater reaction would be expected after the second injection, the host now being immune. Instead, no nodules were formed, thus indicating that, if a chick is previously immunized, the injected lymphocytes are not able to cause a reaction. It would therefore seem that a strong secondary host reaction eliminates the relatively small amount of injected cells without producing any visible reaction, whilst a weak primary host reaction allows the injected cells a chance to react and so to form a nodule. In addition, if the reaction was one of the host against the graft, it should occur upon the injection of monocytes or thymocytes, both of which are antigenic and can induce tolerance (Terasaki, 1959). However, neither monocytes nor thymocytes produced skin nodules.

Returning to the problem concerning the type of cell which causes the graft against host reaction, evidence that monocytes do *not* cause the reaction will be reviewed first. In tissue cultures of chicken blood only the monocytes are thought to survive and reproduce (Carrel & Ebling, 1922; Lewis, 1925). After about 5–7 days lymphocytes are no longer viable (Hall & Furth, 1938; Medawar, 1940; Yoffey & Courtice, 1956). When monocytes cultured for 1–7 days were injected into embryos, no splenic enlargement was produced. Dosages of cells up to 620,000 were injected. If monocytes are responsible for the splenic enlargement caused by diluted blood or lymphocyte preparations, then as few as 500 monocytes should have caused enlargement. The failure of high dosages of cultivated monocytes to cause enlargement carries weight only if we assume that tissue-cultured monocytes are viable and as effective as blood monocytes. That the monocytes are, indeed, viable after tissue culture and trypsinization was shown by their ability to pick up neutral red dye vitally and to grow *in vitro* when recultured. The monocytes could also induce tolerance to skin homografts (Terasaki, 1959), showing that they had not been greatly altered antigenically, and that they were almost certainly alive, since tolerance has not yet been induced by nonliving cells (Billingham, Brent, & Medawar, personal communication; Terasaki, 1959). It is more difficult to show that any antibody-forming capability of monocytes was not altered by tissue culture. However, it can be said that compared with the number of blood monocytes contaminating the lymphocyte suspensions, about 1,000 times as many cultured monocytes did not cause splenic enlargement. Furthermore, monocytes cultured for only one day did not cause splenic enlargement or the development of skin nodules. It is important to note that although monocytes cultured for one day did not produce a skin nodule, the cells *not* adhering to the clot in the same cultures did cause definite nodule development. In this experiment the only difference in treatment of the cells attached to the plasma clot and the cells of the supernatant was that the cells on the plasma clot were treated with trypsin to disengage them. Trypsin treatment itself was shown not to affect activity since trypsinized and untrypsinized lymphocytes produced reactions of equal severity.

The main evidence in favour of the hypothesis that cells of the lymphocyte family are responsible for the graft against host reaction is that blood lymphocytes 99–100 per cent. pure produced marked splenic enlargement. As few as 30,000 lymphocytes have caused detectable splenic enlargement. The degree of splenic enlargement is directly proportional to the number of lymphocytes injected (Text-fig. 1). Lymphocytes also regularly produced skin nodules, while monocytes and thymocytes did not. Splenic enlargement due to slight contamination with monocytes can be eliminated by the considerations mentioned above. If it is true, as most authorities believe, that the 'small lymphocyte' is incapable of division, then the offending cell must be, or must at least stem from, a 'large lymphocyte', for Simonsen's (1957) evidence shows that the causative agent multiplies in the host. However, contamination of the blood preparations by cells such as plasma cells, &c., and hypothetical 'transformation' to cells of other types, cannot be ruled out.

It is interesting to note that thymocytes did not produce a marked splenic enlargement or skin nodule. This is in conformity with much work indicating that thymocytes, though morphologically similar to lymphocytes, do not have the same immunological activity (Yoffey & Courtice, 1956).

SUMMARY

1. An attempt has been made to identify the cell responsible for the splenic enlargement caused by the injection of adult homologous blood and spleen cell suspensions into embryonic or newborn chicks.

2. Adult chicken blood lymphocytes 99–100 per cent. pure produced marked splenic enlargement when injected intravenously into chick embryos of a different breed or strain. As little as 30,000 lymphocytes could produce a detectable effect. Adult chicken spleen-cells also produced enlargement of the embryonic spleen. Adult monocytes and thymocytes did not cause significant enlargement.

3. Evidence is presented that the development of inflammatory nodules after the local intracutaneous injection of cells from adult homologous donors is, like splenic enlargement, principally the result of a reaction of the grafted cells against the host. Adult blood lymphocytes when injected intracutaneously were shown to cause the production of a nodule in 3–5 days. Monocytes and thymocytes did not cause the formation of these nodules.

4. From these experiments it is concluded that, in chickens, cells of the peripheral blood lymphocyte family are immunologically competent, and are capable of producing an *iso*-immune reaction against antigens present in their embryonic or juvenile host.

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Tolerance of Skin Grafts produced by Various Adult Cells, Soluble Extracts, and Embryonic Cells

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INTRODUCTION

AT the present time there are two principal experimental means by which animals can be induced to accept homografts. First, tolerance to homografts can be induced by injection of cells from the future homograft donor into embryonic or neonatal animals (Billingham, Brent, & Medawar, 1956; Billingham & Brent, 1957). Second, successful homografts can be made in a fair percentage of animals which have been rescued from an otherwise lethal irradiation by the injection of bone-marrow or splenic cells from the homograft donor (Main & Prehn, 1955, 1957; Trentin, 1956, 1957). Recently, it has been shown that in both these types of experiments the injected cells often react immunologically against the host, so causing a homograft reaction in reverse. This 'graft against host' reaction in embryonic animals has been studied by Billingham & Brent (1957, 1959), Billingham (1958), Simonsen (1957), and Cock & Simonsen (1958). The 'secondary disease' and delayed death following the injection of homologous bone marrow into x-irradiated recipients have been ascribed in a large measure to a graft against host reaction by various workers (Loutit, 1955; Trentin, 1956, 1957, 1958; Uphoff, 1958; Ilbery, Koller, & Loutit, 1958).

In view of the presence of an underlying graft against host reaction in both of these experimental means by which homografts have been caused to be successful, it is of some importance to know whether it is possible to obtain tolerance to homografts without complications of this kind. The use of embryonic cells which are immunologically immature, and which may themselves become tolerant to the host, has yielded significant numbers of tolerant animals without evidence of other than accidental mortality (Billingham *et al.*, 1956; Hasek, 1954; Cannon, Terasaki, & Longmire, 1957). Correspondingly, in the repair of radiation injuries the use of fetal liver tissue has to a large extent prevented the occurrence of 'homologous disease' (Uphoff, 1958).

A second method of inducing tolerance without runt disease is applicable only when inbred animals are available, viz. the induction of tolerance in animals

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of strain *X* or *Y* by *hybrid X/Y* tissues (Billingham & Brent, 1959). Under these circumstances the hybrid cells cannot react against the host.

Experiments are reported here in which a third method has been tested, viz. the induction of tolerance by adult cells which are *immunologically* (as opposed to genetically) incapable of reacting against the host. As shown in an earlier paper (Terasaki, 1959), two such cells in the chicken are the monocyte and the thymocyte. The power of these cells to produce tolerance has been compared with that of lymphoid cells, which do react against the host. As a fourth possible approach, attempts were made to see whether tolerance could be induced by cell-free antigenic preparations.

The 'graft versus host' interpretation of runt disease and splenic enlargement clearly depends upon the assumption that iso-antigens are present in the embryonic host which are capable of eliciting a reaction from the adult lymphoid cells injected into it. Some importance therefore attaches to the determination of the stage of embryonic life at which iso-antigens become effectively mature. This problem has been studied by examining the competence of chick embryonic tissues of various ages to produce tolerance after injection into newly hatched chicks of a different breed.

METHODS

The breeds of chickens used in these studies were the inbred *I* and *W* lines,[1] the Rhode Island Red × White Leghorn cross (R × L), and the Light Sussex (LS).

Injection of various adult cells

Diluted adult blood, blood lymphocytes, monocytes, thymocytes, and splenic cells were obtained in the manner described by Terasaki (1959). Table 1 shows the dosages of cells, routes of injection, breed combinations, and ages at which the cells were injected. In most cases the cells were injected late in embryonic development to avoid complications attributable to a severe graft against host reaction.

All chicks were tested for tolerance by transplantation of skin grafts from the original tissue donor or from a chicken isologous with the original tissue donor. The grafts were observed up to 30 days after grafting.

Injection of soluble extracts

The technique of isolation of antigens was essentially that described by Billingham, Brent, & Medawar (1958). Briefly, the method consisted of first lysing splenic cells by washing successively in 0·15 M NaCl, 0·05 M NaCl, and water. The sediment was resuspended in water with a piston-and-cylinder type of blendor and exposed to mild ultrasonic vibrations for 60 seconds. The precipitate which formed upon the restoration of the NaCl concentration to 0·15 M was discarded and the supernatant centrifuged for 60 minutes at 25,000 g. The supernatant was discarded, and the antigenically active sediment resuspended in Ringer-phosphate. Intravenous injection of this extract into embryos or into newly hatched chicks led to immediate death. It was therefore injected either intraperitoneally or subcutaneously, as indicated in Table 1. All the newly hatched chicks so injected were skin-grafted on the same day with skin isologous or autologous with the original donor of the splenic extract. The dosage per chick of antigenic extract in wet weight equivalent of spleen (allowing for a 15 per cent. loss in preparation) was as follows: experiment No. 1, 500, 250, or 125 mg.; experiment No. 2, 210 mg.; experiment No. 3, 500 mg.; experiment No. 4, 720 mg. and 720 mg. after storage for 24 hours at 4° C.; experiment No. 5, 1,674 mg. followed 4 days later by 2,300 mg. In experiment No. 5 the extract was prepared from the spleens of 9 *W* line chickens for the first injection and 8 *W* line chickens for the second injection.

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[1] These lines were made available to us by the Northern Breeding Station through the kindness of the Agricultural Research Council; they were developed by Dr. A. G. Cock.

TABLE 1

Tolerance of skin grafts produced by various adult cells and by soluble extracts

		Breed			*Survival of homografts*					
					Partial graft survival			*Complete graft survival*		
Cells injected	*Route of injection*	*Donor*	*Recipient*	*Age at injection*	*14 days*	*20 days*	*30 days*	*14 days*	*20 days*	*30 days*
Adult blood 0·1 c.c.:										
1:3 dilution	i.v.	LS	R × L	14-day incub.	3/3	3/3	3/3	2/3	1/3	0/3
1:9 ,,	i.v.	LS	R × L	,,	6/6	6/6	6/6	5/6	4/6	2/6
1:27 ,,	i.v.	LS	R × L	,,	5/5	5/5	4/5	4/5	2/5	0/5
1:81 ,,	i.v.	LS	R × L	,,	4/5	2/5	0/5	0/5	0/5	0/5
1:243 ,,	i.v.	LS	R × L	,,	5/7	2/7	0/7	0/7	0/7	0/7
1:729 ,,	i.v.	LS	R × L	,,	3/6	2/6	0/6	0/6	0/6	0/6
Blood lymphocytes, 1–3 million	i.v.	*I*	R × L	19-day incub.	13/13	13/13	11/13	9/13	8/13	2/13
Blood lymphocytes, 12–25 million	i.v.	*W*	R × L	1-day post-hatch	7/7	7/7	7/7	7/7	6/7	4/7
Monocytes, 390,000, cultured 6 days	i.v.	*W*	R × L	14-day incub.	5/5	5/5	1/5	1/5	1/5	0/5
Monocytes, 0·5–1·1 million, cultured 8 days	i.v.	*W*	R × L	19-day incub.	9/9	9/9	5/9	7/9	7/9	2/9
Thymic cells, 3 million	i.v.	*I*	R × L	,,	8/8	8/8	6/8	4/8	1/8	1/8
Spleen cells, 28 million	i.p.	LS	R × L	12–24 hours post-hatch	9/11	7/11	5/11	4/11	3/11	2/11
Soluble splenic extract:										
Experiment No. 1	i.p.	R × L	LS	,,	1/15	1/15	0/15	0/15	0/15	0/15
Experiment No. 2	i.p.	LS	R × L	,,	7/9	1/9	0/9	0/9	0/9	0/9
Experiment No. 3	i.p.	LS	R × L	,,	6/8	1/8	0/8	0/8	0/8	0 8
Experiment No. 4; 2 injections, 1 day apart	subcut.	*W*	R × L	,,	3/3	1/3	0/3	0/3	0/3	0/3
Experiment No. 5; 2 injections, 4 days apart	i.p. and subcut.	*W*	R × L	,,	2/4	0/4	0/4	0/4	0/4	0/4
Controls		LS	R × L	,,	14/19	2/19	0/19	0/19	0/19	0/19
Controls		*W*	R × L	,,	6/8	0/8	0/8	0/8	0/8	0/8

Preparation of cell suspensions from embryonic chicks

Cell suspensions were prepared from the spleens or kidneys, hearts, and lungs of 5 19-day- and 14 13-day-old embryos. The tissues were broken up in a small glass piston blender, the clumps allowed to settle, and the cells taken from the supernatant fluid. Preparations of cell suspensions from 22 6-day- and 39 4-day-old embryos were made from the whole body of the embryos. The embryos were broken up by pipetting in Ringer-bicarbonate and digested for 30 minutes at 37° C. with 0·5 per cent. of 'Difco' trypsin '1:250'. About 0·1 mgm. of DNA-ase was then added to break up the nucleoprotein gel and, after 30 minutes incubation at 37° C., the cells were washed in heparinized Ringer-phosphate (heparin: 25 u./ml.). In order to obtain a single cell suspension, the supernatant fluid was cropped four times after very light centrifugation for 5 minutes at about 300 r.p.m.

These suspensions were injected intravenously into newly hatched chicks in the dosages and strain combinations indicated in Table 2. All the injected chicks were skin-grafted from an isologous adult chicken on the same day.

RESULTS

Table 1 shows the incidence of tolerance to skin homografts produced by injections of various cells. Adult whole blood diluted up to 1:27 induced tolerance (the grafts of 4/5 subjects showing some degree of survival at 30 days), whereas blood diluted 1:81 and beyond did not (0/18). Blood lymphocytes produced a marked degree of tolerance (18/20), and a measurable degree of tolerance was induced by monocytes (6/14), thymocytes (6/8) and splenic cells after intraperitoneal injection (5/11). The duration of tolerance was not tested

TABLE 2

The induction of tolerance to adult skin homografts by embryonic cells of various ages

Incubation age of donor embryo (days)	*Type of cells injected*	*No. of cells per bird* ($\times 10^6$)	*Donor breed*	*Recipient breed*	*Survival of homografts*					
					Partial graft survival			*Complete graft survival*		
					14 days	*20 days*	*30 days*	*14 days*	*20 days*	*30 days*
19	kidney, heart, and lung or spleen	2–5	*W*	R×L	8/8	7/8	7/8	3/8	3/8	1/8
13	blood or spleen	2–5	*I* or *W*	R×L	13/13	12/13	9/13	9/13	5/13	1/13
6	blood or whole body	25	*I* or *W*	R×L	10/15	10/15	5/15	0/15	0/15	0/15
4	whole body	17	*I* or *W*	LS	9/12	8/12	2/12	2/12	1/12	1/12
Controls	None	..	*W*	R×L	3/11	0/11	0/11	0/11	0/11	0/11
Controls	None	..	*I*	R×L or LS	1/6	0/6	0/6	0/6	0/6	0/6

longer than 30 days, but in the few chicks allowed to go further the skin grafts were sloughed in the second month.

As is evident from Table 1, soluble extracts prepared from spleens produced uniformly negative results, whether after single or repeated injections (0/39). At no stage did animals which received splenic extract differ significantly from the control animals which received no treatment at all (0/27 surviving homografts at 30 days).

In the experiments designed to determine the earliest age at which embryonic tissues possess antigens capable of inducing tolerance to homografts, even 4-day chick embryo tissues were shown to induce some degree of tolerance (Table 2). Thus, at 20 days, when there were no surviving grafts in 17 controls, 8/12 chicks injected with 4-day embryo tissues still retained surviving grafts. Tissues from older embryos induced a degree of tolerance which increased with the age of the tissue: taking survival at 20 days as the criterion for tolerance, 6-day embryo tissues induced tolerance in 10/15, 13-day tissues in 12/13, and 19-day tissue in 7/8 chicks.

DISCUSSION

In the preceding paper (Terasaki, 1959) blood lymphoid cells were shown to cause a splenic enlargement attributable to a graft against host reaction, whereas monocytes and thymocytes caused no such enlargement. In the results described here, monocytes and thymocytes induced tolerance to homografts almost as effectively as blood lymphoid cells. Thus it is clear that cells capable of inducing tolerance to homografts can be distinguished from cells responsible for the graft against host reaction. The graft against host reaction can apparently be avoided by use of tissues free of lymphoid cells.

Lymphoid cells themselves, although capable of causing a graft against host reaction, can elicit tolerance to homografts without overt or clinical signs of a graft against host reaction. But this is only true if the lymphocytes are injected late in the development of the chick, i.e. on the threshold of that stage at which the chick changes over to becoming reactive against homografts. It seems probable that the lymphocytes get into the animal early enough to induce tolerance, but too late to damage the host fatally. Injections of lymphocytes earlier in life, or in higher dosages, are known to have a deleterious or fatal effect.

Previous attempts to induce tolerance to skin grafts in mice with cell-free antigenic extracts have been unsuccessful (Billingham, Brent, & Medawar, personal communication). The chick seemed to be an ideal animal to reinvestigate the problem since in the chick it is possible both to inject soluble extracts and to graft skin on the day of birth. Thus a minimum of time elapses between first exposure to the antigenic stimulus and the transplantation of the skin graft that is used to test for tolerance. In spite of this advantage, no detectable degree of tolerance was found in five separate trials—even with a second injection of antigenic extract 4 days after the first. Unfortunately, the extract could not be injected intravenously because this led to almost immediate death of chicks and chick embryos. Intraperitoneal injection, however, should have been at least partially effective, since it was shown that intraperitoneal injection of spleen cells induced tolerance. Whether the inability of the extracts to induce tolerance is due to the lack of certain antigens in the preparation, or to the antigens being in unsuitable form, or to other causes, remains to be determined.

Although much work has been done on the question of whether fetal homografts will 'take' better than adult grafts (e.g. Baxter *et al.*, 1958; Toolan, 1958; Kooreman & Gaillard, 1950; May, 1953; Terasaki, Longmire, & Cannon, 1957) little systematic study has been made of the age at which adult transplantation antigens appear during development. Antigenic analysis of embryonic tissue by the ordinary immunity test has been hindered by the difficulty that the embryonic cells would grow and presumably mature during the 1–2 week period in which they induce immunity to their antigens. This difficulty was overcome by testing the power of embryonic cells to induce *tolerance* in newly hatched chicks. Such chicks are known to be near the end of the period in which they can be rendered tolerant (Billingham, Brent, & Medawar, 1956). The injected cells must therefore exert their effect within a few days, and any maturation of the cells after that time will not be effective in inducing tolerance. With such tests, embryonic chick tissues from as early as the 4th day of development produced a detectable degree of tolerance. Adult antigens are therefore present very early in development. Although older embryos have induced a greater degree of tolerance, it is difficult to conclude from this that there is a gradual development of adult antigens during development, as it was not technically feasible to compare the effect of any one kind of tissue from embryos of different ages.

SUMMARY

1. Tolerance of skin homografts was studied after the injection of various adult cells and soluble tissue extracts into newly hatched or embryonic chicks. Tolerance (measured by the proportion of surviving skin homografts 30 days after transplantation) was induced in 50 per cent. or more of the chicks injected with diluted whole blood, blood lymphocytes, blood monocytes, and thymocytes. Since monocytes and thymocytes do not produce a 'graft against host' reaction it follows that tolerance to homografts can be induced by adult cells without the complications associated with runt disease.

2. Tolerance could not be induced with cell-free extracts of known antigenic potency.

3. Analysis by the tolerance test of the appearance of adult-type iso-antigens during embryonic development shows that they are detectable by the 4th day of embryonic life.

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Hair Growth and its Relation to Vascular Supply in Rotated Skin Grafts and Transposed Flaps in the Albino Rat

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WITH THREE PLATES

INTRODUCTION

HAIR-GROWTH in the rat occurs in a series of waves which start ventrally and pass over the flanks to the back (Dry, 1926; Butcher, 1934). In our colony of randomly mated albino rats the first wave begins at about 5 weeks of age and is complete 3 weeks later; the second wave starts at $8\frac{1}{2}$–9 weeks of age and is not complete until a further 12–14 weeks have elapsed. In both these waves dorsal hairs are longer than ventral ones, and this appears to be due to a longer growing period rather than to any differences in the rate of growth (Johnson, 1958*a*). Haddow & Rudall (1945) demonstrated that such waves of growth are accompanied by increased vascularization of the dermis, and suggested that follicles become active as a response to increased vascularity. Durward & Rudall (1949) seem to have modified this view to the extent of suggesting that vascularization cannot cause follicular activity until a required resting stage has been completed.

Our experiments involved the transplantation or transposition of hair follicles, i.e. removing them from their original blood-supply and allowing them to develop vascular connexions in their new sites. First, we wanted to discover whether the activity of the hair follicle is consequent upon, or in fact initiates, vascularization. Secondly, we wanted to know whether short ventral and longer dorsal hairs are characteristic of their respective follicles, or whether the hair-length merely depends on the site of the follicle.

Previous investigators have reached differing conclusions. Butcher (1936) believed that transplanted follicles behave according to their new position (i.e. sitewise), although his pioneer work is not easy to interpret because the experiments were not designed in the light of a detailed knowledge of the hair-growth cycles. Durward & Rudall (1949), who twisted lateral strips of skin in rats leaving the blood-supply as intact as possible, and Ghadially (1957) who, in the rabbit, devised an ingenious transposition of skin flaps with their original connexions

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retained along one border, concluded that the follicles retain an inherent rhythm (i.e. they behave selfwise). We do not think these experiments were definitive, because in neither case was the original vascular supply cut off.

Our work will be presented in two sections. The first deals with the activity of hair follicles in grafts and transposed flaps as studied in living rats, and the second with their vascular supply as studied in histological sections.

HAIR-GROWTH IN ROTATED SKIN GRAFTS AND TRANSPOSED FLAPS

Methods

Animals

All the observations were made on albino rats from a randomly mated colony.

Design of experiments

Groups of three litter-mates were used either for skin grafts or transposed flaps.

Skin grafts. In one rat of each group a skin graft was cut and stitched back into position at 4 weeks of age; in another a similar graft was rotated; a third rat served as an intact control.

Transposed flaps. In one rat of each group skin flaps were transposed; in another the skin was cut but not transposed; a third rat served as an intact control.

Before operation all the rats were shaved and dyed black with a commercial hair-dye, 'Inecto' (Rapidol Ltd.), so that newly growing white hairs could easily be seen.

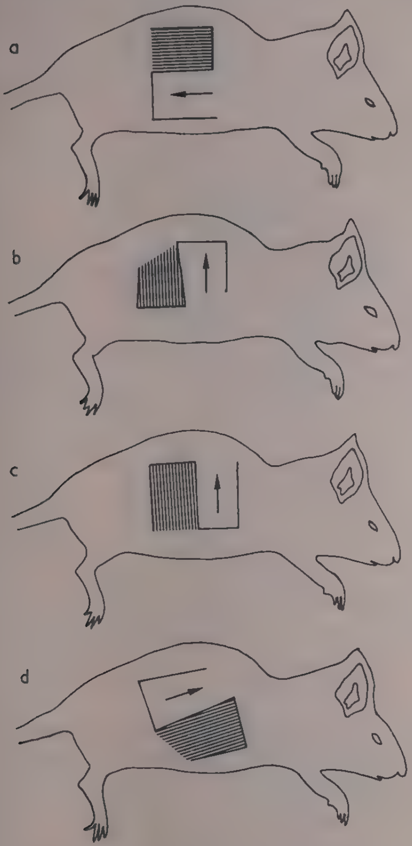
Operative procedures

Skin grafts. A strip of skin from the flank (about 4 × 2 cm.), extending from the belly to the back was separated above the level of the panniculus carnosus, either replaced or rotated through 180°, and carefully stitched into position with interrupted sutures of fine silk using ophthalmic needles.

In general, the methods used were similar to those described by Medawar (1944). Instruments and silk were sterilized in 'Novasapa' (Pharmaceutical Manufacturing Co.). The rats were under ether anaesthesia; before operation the hair was clipped and the skin cleaned with a dilute antiseptic, 2 per cent. 'Dettol' (Reckitt & Colman). Subsequently the grafts were dusted with sterile sulphadiazine, dressed with sterile vaseline gauze, followed by a sterile gauze pad and a few turns of bandage. Finally, the body was enclosed in a tube of plaster bandage. The dressings and stitches were removed after about 6 days; occasionally it was found necessary to put on new dressings for a few days.

Transposed flaps. The initial operation, carried out at 4 weeks of age, was similar to that devised for the rabbit by Ghadially (1957). Dorsal and ventral flaps were separated by an incision as shown in Text-fig. 1*a* and in Plate 1, fig. A.

The ventral flap was then turned dorsally and forwards, and the dorsal flap ventrally and backwards, to occupy the positions shown in Text-fig. 1*b* and Plate 1, fig. B; the flaps were then sutured in position. The methods of suturing and dressing were similar to those already described.



TEXT-FIG. 1. Method of cutting and transposing skin flaps. *a*, flaps cut before making first transposition. The dorsal flap is shaded; the arrow on the ventral flap shows the direction of the grain of the hair (always the same on both flaps at every stage of the transposition). *b*, the flaps after transposing and suturing. *c*, the flaps cut before making the second transposition. *d*, the flaps after transposing and suturing for the second time.

At 7 weeks of age, after the first wave of growth had passed, the rats were once more shaved and dyed and a second operation was carried out. The anterior and posterior flaps were again separated by cuts, but the anterior flap was now severed ventrally and the posterior flap dorsally, along the axes which had previously remained intact. (Text-fig. 1*c*; Plate 1, fig. D). A further transposition was then made (Text-fig. 1*d*; Plate 1, fig. E). The result of this was to bring the original ventral flap into the dorsal position and the original dorsal flap into the ventral position, and also to sever in two stages all the original vascular connexions. The grain of the hair now faced forwards in both flaps.

RESULTS

Passage of growth waves in grafts

In all the grafted rats the first and second waves of hair-growth were initiated and moved over the body at the same time as in the intact rats. Hence the operation did not have any over-all effect on the timing of the waves.

The appearance of hair on the unrotated grafts was well in retard of the wave on the body (Plate 1, figs. G, I). But when hair did start to grow on the graft it appeared first ventrally, and the growth front advanced dorsally in the normal way. It is interesting to note (see Plate 1, fig. J) that when the advancing front of the body-wave reaches the level of the top of the graft, hair starts to grow dorsal to the graft, even though the wave on the graft is still in retard. This suggests, though it does not prove, that the wave is not propagated. The second wave of growth was similarly retarded on the graft, in spite of the fact that some 30 days had elapsed since operation.

There was a similar delay in the eruption of hair on the rotated grafts. When growth started it was much less regular than on the control grafts; at first the hairs appeared to be in isolated tufts. But there was no doubt whatever that the first hairs appeared on the dorsal part of the graft and that the waves of growth spread from dorsal to ventral regions (Plate 1, fig. H).

TABLE 1

Eruption of hairs of second growth-wave (in days after birth)

	Intact rat	*Rat with unrotated graft*				*Rat with rotated graft*			
Litters	(*a*) *Ventral body*	(*b*) *Ventral body adjacent to graft*	(*c*) *Dorsal body adjacent to graft*	(*d*) *Ventral part of graft*	(*e*) *Dorsal part of graft*	(*f*) *Ventral body adjacent to graft*	(*g*) *Dorsal body adjacent to graft*	(*h*) *Ventral part of graft*	(*j*) *Dorsal part of graft*
Males									
1	57	—	—	—	—	57	61	93	72
2	58	58	79	73	83	58	84	83	80
3	65	58	72	69	94	58	70	88	79
4	65*	58	72	73	88	58	69	100	88
5	58	57	81	66	85	57	76	105	78
Females									
1	59	59	74	66	92	—	—	—	—
2	64	—	—	—	—	61	72	79	69

* Same control rat as in litter 3 (experimental litters 3 and 4 contained rats from one large litter).

The above descriptions apply without exception to all the experiments carried out. Some rats were discarded because the grafts or the animals became unhealthy; five control grafts and six rotated grafts survived successfully and were used for detailed observations on the rate of passage of the waves and on the lengths of hair produced.

The first wave of growth passes rapidly and was usually well under way by the time the dressings were removed from the rats. More detailed observations were possible on the second wave, and these will be considered first.

The time of eruption of the hairs on the body and on the grafts is recorded in Table 1, from which the following facts may be induced.

(i) The operation of skin grafting does not affect the time of eruption of the ventral hairs on the body adjacent to the graft (columns (*a*), (*b*), and (*f*)).

(ii) In unrotated grafts the time of eruption of the ventral hairs is delayed (columns (*b*) and (*d*)).

(iii) In unrotated grafts, as in intact rats, the ventral hairs erupt before the dorsal ones (columns (*d*) and (*e*)).

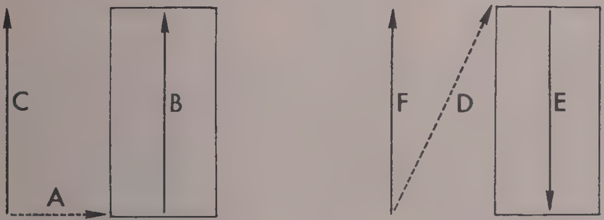
(iv) In the rotated grafts the hairs erupt dorsally before they do ventrally (columns (*h*) and (*j*)).

It would be interesting to know whether rotation of grafts affects the timing of the first appearance of hair, as well as to compare the rates of passage of the hair-wave in normal skin, unrotated grafts, and rotated grafts. Table 2, constructed from the data of Table 1, attempts to throw light on these problems; the meaning of columns A to F is explained in Text-fig. 2. It seems likely that the second wave is more delayed in the rotated grafts than in the unrotated ones (see male litters 2–5, columns A and D). Although the figures show wide variation, there appears to be no reason for supposing that the time of passage of the growth-wave is altered by grafting or by rotating.

TABLE 2

Delay, due to grafting, in eruption of hairs and times of passage of second wave of hair-growth in unrotated and rotated skin grafts (in days)

	Unrotated grafts			*Rotated grafts*		
Litters	*A* *Delay of start of hair growth*	*B* *Time of passage on graft*	*C* *Time of passage on body adjacent to graft*	*D* *Delay of start of hair growth*	*E* *Time of passage on graft*	*F* *Time of passage on body adjacent to graft*
Males						
1 . .	—	—	—	15	21	4
2 . .	15	10	21	22	3	26
3 . .	11	25	14	21	9	12
4 . .	15	5	14	30	12	11
5 . .	9	19	24	21	27	19
Females						
1 . .	7	26	25	—	—	—
2 . .	—	—	—	8	10	11



TEXT-FIG. 2. Diagram to explain data given in Table 2. Continuous arrows represent time taken for movements of hair-growth front in unrotated graft (B), rotated graft (E), and adjacent body (C, F). Broken arrows (A, D) represent delay between appearance of hair on body and appearance of hair on graft.

The passage of the first wave of growth (Table 3) was much more rapid than the second, although it followed a similar pattern in the respective grafts.

TABLE 3

Eruption of ventral and dorsal hairs of first growth-wave (in days after birth)

	Unrotated grafts		*Rotated grafts*	
Litter	*Ventral part of graft*	*Dorsal part of graft*	*Ventral part of graft*	*Dorsal part of graft*
Males				
1	—	—	53	44
2	46	51	51	46
3	45	48	51	48
4	47	48	47	45
5	39	45	44	41
Females				
1	53	56	—	—
2	—	—	48	45

Passage of growth-waves in transposed flaps

After the initial transposition, the first wave of hair-growth invariably moved into the anterior flap before it moved into the posterior flap (Plate 1, fig. C). There appeared to be no delay in passage of the wave into the transposed flaps; the wave passed at the same time, as it moved from ventral to dorsal, in the intact litter mates and in the operated control rats. After the second transposition, hairs first appeared on the dorsal flap at the same time as on the ventral body (Plate 1, fig. F), followed by simultaneous growth on the ventral flap and the dorsal body (Table 4).

TABLE 4

Eruption of hairs of second wave on control and doubly transposed flaps (in days after birth)

	Cut control flaps		*Transposed flaps*		
Litter	*Ventral body and ventral flap*	*Dorsal flap*	*Ventral body*	*Dorsal flap*	*Ventral flap*
Males					
1	60	63*	60	63	71
2	60	70	60 60	— 61	— —
Females					
1†	60	64‡	74	74	64
2	63	70	63 61	64 63	— 66

* Lower part only. Growth had not occurred throughout the flap until 70 days.

† In this litter there appears to be an anomaly in the time of eruption of hairs in the rat with transposed skin flaps. The ventral body-hair did not appear until about 10 days later than the expected time, although this was coincident with eruption of hairs on the dorsal flap. The hairs which appeared on the ventral flap at 64 days may have been delayed growth from the first wave.

‡ Lower part.

TABLE 5

Definitive lengths of hair (mm.) of second growth-wave

The means and standard errors are for 20 hairs clipped at skin level from each region

Litter		*Unrotated graft*	*Body opposite graft*	*Rotated graft*	*Body opposite graft*
Males					
1	Ventral	—	—	11·7±0·38	10·4±0·19
	Dorsal	—	—	9·7±0·16	13·4±0·52
2	Ventral	7·0±0·17	6·8±0·13	12·4±0·31	6·5±0·20
	Dorsal	14·4±0·51	12·5±0·35	5·6±0·17	13·4±0·39
3	Ventral	8·6±0·19	8·6±0·26	12·4±0·28	9·4±0·14
	Dorsal	12·2±0·35	12·5±0·29	10·3±0·16	12·3±0·27
4	Ventral	7·7±0·27	6·6±0·17	9·5±0·16*	7·0±0·18
	Dorsal	12·8±0·20	12·7±0·27	6·6±0·16	12·4±0·45
5	Ventral	8·4±0·19	7·7±0·14	12·5±0·23	6·0±0·21
	Dorsal	14·7±0·27	11·8±0·50	7·2±0·18	13·1±0·46
Females					
1	Ventral	9·7±0·21	10·0±0·19	—	—
	Dorsal	11·9±0·40	11·8±0·16	—	—
2	Ventral	—	—	10·9±0·41	6·7±0·15
	Dorsal	—	—	6·7±0·27	12·6±0·39
3	Ventral	9·4±0·19	8·4±0·23	—	—
	Dorsal	11·6±0·36	11·4±0·28	—	—

* Graft hairs clipped before fully grown.

TABLE 6

Hair lengths (mm.) of first wave

The averages are for 20 hairs clipped at skin level in each region when the rats were 7½ weeks of age

Litter		*Unrotated graft*	*Body opposite graft*	*Rotated graft*	*Body opposite graft*
Males					
1	Ventral	—	—	9·4	4·8
	Dorsal	—	—	7·2	10·0
2	Ventral	—	—	7·6	5·6
	Dorsal	—	—	6·2	10·9
3	Ventral	5·5	5·4	4·8	5·2
	Dorsal	8·6	10·8	2·9	9·8
4	Ventral	3·9	5·2	7·2	5·6
	Dorsal	7·1	9·6	5·3	9·8
5	Ventral	9·4	9·2	10·2	5·6
	Dorsal	10·4	10·9	5·1	10·7
Females					
2	Ventral	—	—	8·4	4·8
	Dorsal	—	—	4·9	10·8

Length of hairs in grafts and transposed flaps

On the unrotated grafts, the ventral and dorsal hairs attained lengths similar to those of hairs on the opposite flank (Table 5). On the rotated grafts, the length of hair produced was in every case consistent with the origin of the follicles and not in accordance with their new position. Very similar results were seen in the shorter hairs of the first wave (Table 6), although these were clipped at 7½ weeks and may not all have been fully formed.

TABLE 7

Mean definitive hair lengths (mm.) of second wave in control and doubly transposed flaps

The terms 'ventral' and 'dorsal' refer to the new sites of the flaps (see Text-fig. 1)

Litter		*Control flaps*	*Body opposite flaps*	*Transposed flaps*	*Body opposite flaps*
Male					
1	Ventral	7·3±0·35	6·2±0·15	11·4±0·22	9·5 ±0·33
	Dorsal	12·3±0·40	13·3±0·39	10·2±0·29	13·0±0·31
Females					
1	Ventral	6·9±0·26	6·2±0·16	11·0±0·24	8·3±0·21
	Dorsal	10·6±0·18	10·7±0·27	7·2±0·21	11·0±0·29
2	Ventral	5·9±0·14	5·5±0·14	11·9±0·34	6·6±0·12
	Dorsal	10·8±0·26	10·1±0·33	7·9±0·21	10·7±0·30
2	Ventral	—	—	10·9±0·23	6·9±0·18
	Dorsal	—	—	6·6±0·11	10·9±0·27

The average length of the fully grown hairs on the transposed flaps is shown in Table 7. Although figures are only available for four rats, it is quite clear that the hair-lengths on the transposed flaps are those characteristic of their follicles and not of the site to which they have been moved.

VASCULAR SUPPLY OF ROTATED SKIN GRAFTS AND TRANSPOSED FLAPS

Methods

Materials

Rotated and unrotated grafts, or transposed skin flaps with operated controls, were performed as described in the previous section. Operated rats of three litters were killed for examination at each of the following stages:

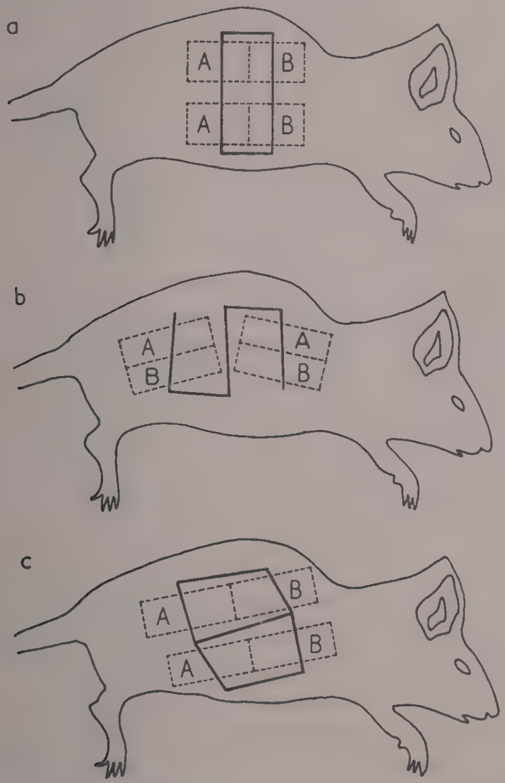
(i) 5 days after grafting.
(ii) 10 days after grafting.
(iii) At the first appearance on the grafts of hairs of the first growth-wave.
(iv) At the first appearance on the grafts of hairs of the second growth-wave.
(v) After the initial transposition, at the first appearance on the flaps of hairs of the first wave.

(vi) After the second transposition, at the first appearance on the flaps of hairs of the second wave.

Intact litter mates were killed either at the same time ((i), (ii)) or when the growth-waves had reached the flank ((iii), (iv), (v), (vi)).

Histological methods

The rats were killed with chloroform and skinned from a mid-ventral incision, and the whole skins were wetted with alcohol, fastened to cork boards, and fixed in 10 per cent. formalin/hypertonic saline (Pickworth, 1934) for 24 hours.



TEXT-FIG. 3. Showing the regions from which samples were taken for histological study after grafting or transposition of flaps. *a*, rotated or unrotated graft; samples removed at stages (i), (ii), (iii), and (iv) (see p. 424). *b*, flaps after initial transposition; samples removed at stage (v). *c*, flaps after second transposition; samples removed at stage (vi). Samples marked A were used for demonstration of blood-vessels; collateral samples marked B were embedded in ester wax for general histology.

Samples of both dorsal and ventral regions of the grafts, or of both dorsal and ventral skin of the flaps, each together with some adjacent body-skin, were soaked in gum phenol for 2 days, frozen and sectioned sagittally at about 150 μ, and the blood-vessels stained as described by Pickworth (1934). Haemoglobin, but not the vessel wall, is stained black by this method; thus small vessels probably remain undetected. Collateral samples were embedded in ester wax (Steedman, 1947), sectioned at 7 μ and stained in haematoxylin and eosin. The exact relationship of the samples to the grafts or flaps is shown in Text-fig. 3. In the intact rats of each litter, samples were taken from the mid-flanks and treated as above.

RESULTS

Skin grafts

Skin samples from the mid-flank region of 33-day-old intact rats (i.e. litter mates of rats killed at stage (i), 5 days after grafting) contained follicles in down-growth—indicating the beginning of the active period; a vascular supply to these follicles was not yet well developed. Five days later (in litter mates of rats killed at stage (ii), 10 days after grafting) growing hairs had erupted, and the follicles were surrounded by a well-developed network of blood-vessels. Thus it seems that in intact rats the start of follicular activity precedes vascularization.

Both rotated and unrotated grafts of 5 or 10 days ((i), (ii), see p. 424) contained only resting follicles without demonstrable vascularization. However, in the adjacent body-skin both follicular activity and vascularization were as developed as in the intact litter mates. Resting follicles of graft skin adjacent to active, well vascularized follicles of body-skin 10 days after grafting can be seen in Plate 2, fig. K.

The appearance of hairs of the first wave on unrotated or rotated grafts (iii) was accompanied by vascularization of the follicles; in the adjacent body-skin the active phase was not yet complete and the follicles were still vascularized.

In unrotated grafts, hairs of the second wave first erupted ventrally (iv), the follicles becoming well vascularized. At the same time the follicles of the adjacent body-skin were nearing the end of activity and the vascular supply was reduced. Dorsally the graft follicles were still resting and poorly vascularized, whilst the follicles of the adjacent body-skin were active and fully vascularized.

In the rotated grafts, active, well vascularized follicles first appeared in the dorsal region (iv). The follicles of the adjacent body-skin were either reaching the end of activity (catagen) and vascularization was decreasing, or they were in the resting stage with no demonstrable vascular network. Such a juxtaposition of active, vascularized graft follicles and resting body follicles is seen in Plate 3, figs. L, M. The ventral follicles of such a graft were still producing hairs of the first wave, although the adjacent body follicles had reached the resting stage of the second wave.

Transposed flaps

The histology and vascular supply of the follicles of the control flaps were indistinguishable from those of the adjacent body-skin.

In samples taken after the single transposition (v), growing follicles with a well-developed blood-supply were present in both the anterior flap and the adjacent ventral body-skin. At this time the follicles in the posterior flap were either resting or beginning downgrowth; in either stage the accompanying blood-supply was poorly developed. In the adjacent ventral body-skin, on the other hand, follicles were active and well vascularized.

After the second transposition (vi), the follicles of the dorsal flap were active and well vascularized; those of the adjacent body-skin were resting and without a discernible blood-supply. In contrast, the follicles in the ventral flap were resting or just beginning downgrowth with only a poorly developed blood-supply, and the follicles of the adjacent body-skin were active and well vascularized.

DISCUSSION

If hair follicles are translocated by rotation of grafts or transposition of flaps they do not take up the rhythm characteristic of their new site, and thus their activity may be out of phase with that of adjacent body follicles.

Our results show clearly that vascularization of follicles in grafts or transposed flaps was always consequent upon their activity; conversely, resting follicles never had a blood-supply demonstrable by a haemoglobin stain even if follicles in adjacent areas were active and well vascularized. In intact rats, moreover, vascularization of follicles could not be demonstrated until after follicular activity had begun.

In both rotated grafts and transposed flaps the follicles produced hairs of a length characteristic of their origin rather than of their new site. Since it is almost certain that dorsal hairs are longer than ventral ones because they grow for a longer period (Johnson, 1958*a*), it seems likely that translocation of follicles does not alter the timing of their active phase.

Grafting, which involves the complete severance of the blood-supply, delays the initiation of follicular activity. Such a delay of the first wave of growth is presumably due to the fact that the graft is not yet established, so that follicular vascularization is impossible. But the fact that there is a similar delay of the second wave suggests that, once re-established, the rhythm of the graft follicles continues regularly and independently.

These observations suggest that the pattern of the waves of hair-growth is inherent in the follicles, and that the hyperaemia which accompanies hair-growth is consequent upon innate demands of the follicles and not the cause of their activity.

It has been previously shown, however, that although various treatments alter the speed at which the waves of hair-growth pass around the body, the pattern is never changed (Johnson, 1958 *b*, *c*); that is to say, adjacent follicles cannot be put out of phase without surgical interference. The simplest explanation of this fact would be that the activity of the follicles is subject to some form of systemic control.

All these different observations might be explained by postulating that follicular activity in different regions of the body is determined by graded local thresholds to a systemic stimulus. Thus follicles would react to the same level of stimulation when translocated as they would have done in their original sites.

If such a hypothesis is correct, one would expect that, once vascularization is re-established, the follicles of unrotated or rotated grafts would become active at the same time as body follicles of similar regions. In fact, after surgical interruptions of the blood-supply, which delays initiation of hair-growth, the activity of the graft follicles continued out of phase. We have to conclude, therefore, that follicular activity depends mainly on an inherent rhythm. Two minor discrepancies remain, namely, that hair-growth is both more delayed and less regular in rotated than in unrotated grafts. These might be explained on the assumption that the blood-supply is more easily re-established when the graft is not rotated. But they could indicate that some sort of dermal gradient plays a small and not overruling part in influencing the pattern of growth.

Whiteley (1958) has recently published the results of transplanting belly-skin to the neck by means of pedicle grafts in the rabbit. He found that the transplanted skin continued to behave as if it had not been removed from the original site; thus his results and ours are in agreement. He did not, in fact, study the vascular supply of the transplants, although he states that it must have been that of the surrounding skin; indeed, on this basis Whiteley concludes that skin has an intrinsic pattern of growth which is not controlled by vascular factors. We agree with these general conclusions, but our results show that vascularization of the follicles is determined by their activity, and is not necessarily similar to that of the surrounding skin.

SUMMARY

1. Hair follicles of albino rats were translocated and allowed to develop vascular connexions in new sites. The translocation was achieved either by rotation of skin grafts in the mid-flank before the expected start of the first wave of growth (4 weeks after birth), or by transposition of flaps—in two stages—so that they were never completely cut off from a blood-supply.

2. The first and second waves of hair-growth on the body adjacent to the grafts or flaps continued as in intact rats; the waves of growth moved from ventral to dorsal, and the dorsal hairs were longer than ventral ones.

3. The appearance of hair of the first wave on unrotated control grafts was in retard of that on the body, but passed from ventral to dorsal in the normal way. During the second wave of growth, follicular activity on the graft continued out of phase with that on the body.

4. The first and second waves of hair-growth on rotated grafts were similarly retarded, but began in the dorsal region and passed ventrally.

5. There was no retardation of growth on the transposed flaps. Hairs first appeared on the dorsally transposed ventral skin of the flaps at the same time as on the ventral body, followed by growth on the ventral flap and dorsal body.

6. The length of hair produced on both rotated grafts and transposed flaps was consistent with the origin of the follicles and not in accordance with their new positions.

7. In intact rats it was demonstrated that follicular activity precedes vascularization.

8. Vascularization of follicles in grafts or transposed flaps was always consequent upon follicular activity, even when this was out of phase with the normal waves of hair-growth on the adjacent body.

9. The evidence suggests that follicular activity depends mainly on an inherent rhythm, and that the hyperaemia which accompanies hair-growth is consequent upon an innate demand of the follicles and is not itself the cause of their activity.

ACKNOWLEDGEMENTS

We wish to express our gratitude to County Laboratories for the donation of a Research Fellowship to one of us (E. J.) and for a generous grant towards the expenses of the work.

We also thank Mr. J. Hancock and Mr. T. Sadler for the photographs and photomicrographs, Mr. J. Skinner for technical assistance, and Mr. N. Smith of Radipol Ltd. for the gift of 'Inecto' hair-dye.

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EXPLANATION OF PLATES

PLATE 1

Transposed flaps

FIG. A. Rat at 33 days of age, showing flaps cut before transposing. The animal was dyed with 'Inecto' and the side shaved before operation.

FIG. B. The ventral flap has now been turned forwards and dorsally; the dorsal flap backwards and ventrally. The flaps are sutured in their new positions.

FIG. C. Seven days later. The dressings have been removed. The first wave of hair-growth has started and has reached the anterior flap (previously ventral) but not the posterior flap. The timing of the wave is not altered.

FIG. D. The same rat at 53 days of age, showing the flaps cut prior to the second transposition. (The rat has again been shaved and dyed.)

FIG. E. The flaps are now transposed so that the anterior flap (originally ventral) is dorsal to the posterior flap (originally dorsal). On both flaps, however, the grain of hair is reversed.

FIG. F. Fifteen days after the second transposition (i.e. at nearly 10 weeks of age) the second wave of growth has reached the middle of the flank. No growth has yet occurred on the ventral flap (originally dorsal skin), but growth is taking place on the dorsal flap (originally ventral skin) at the same time as on the ventral body.

Skin grafts

FIG. G. Rat, age 44 days, 16 days after grafting; this graft was rotated, but unrotated grafts present a similar picture at this stage. The rat was shaved and dyed with 'Inecto' before operation. The new white hair of the first wave of growth has already reached the mid-flank, but there is as yet no growth on the graft.

FIG. H. Rotated graft 7 weeks after operation (i.e. host 11 weeks of age); the second wave of hair-growth on the body has passed by the graft, and hair-growth on the graft has now begun dorsally. (The rat was shaved and dyed at 7 weeks of age.)

FIG. I. Unrotated graft 7 weeks after operation (i.e. host 11 weeks of age); the second wave of hair-growth on the body is approaching the level of the top of the graft, and hair-growth on the graft has now begun ventrally. (The rat was shaved and dyed at 7 weeks of age; body-hair overlying the anterior edge of the graft has been clipped short.)

FIG. J. Unrotated graft; hair of the second wave of growth has now appeared dorsal to the graft even though the wave on the graft is still in retard.

PLATE 2

FIG. K. Sagittal section (thickness about 150 μ) through the dorsal region of a rotated graft together with adjacent body-skin of a rat (38 days of age) 10 days after grafting, showing resting graft follicles adjacent to active body follicles. × 36.

PLATE 3

Sagittal section (thickness about 150 μ) through the dorsal region of a rotated graft (fig. L), together with adjacent body-skin (fig. M) of a rat killed when hairs of the second wave of growth first appeared on the graft (stage (iv)). Active, vascularized graft follicles appear adjacent to resting body follicles. Note that the active skin (of the graft) is thinner than the resting dorsal skin of the body. × 24.

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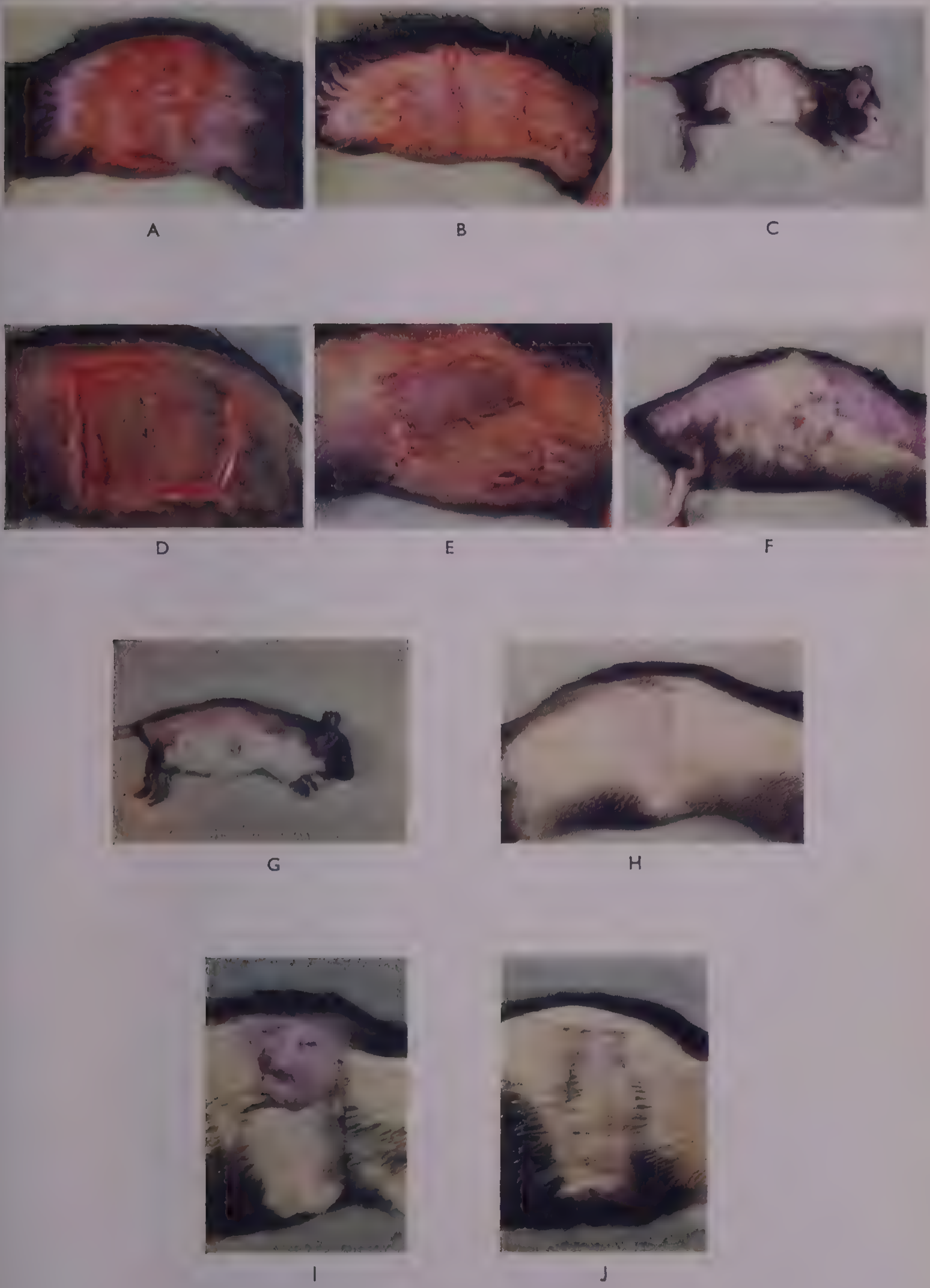
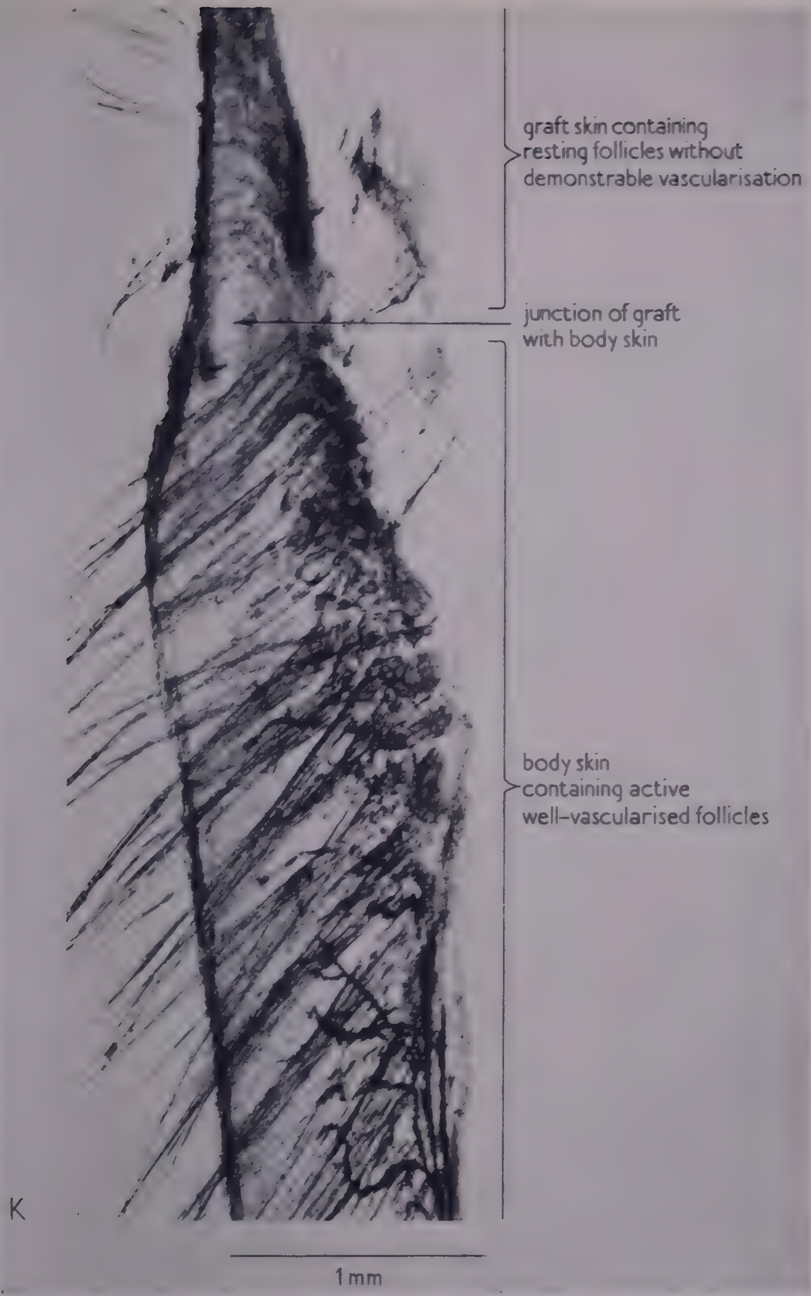


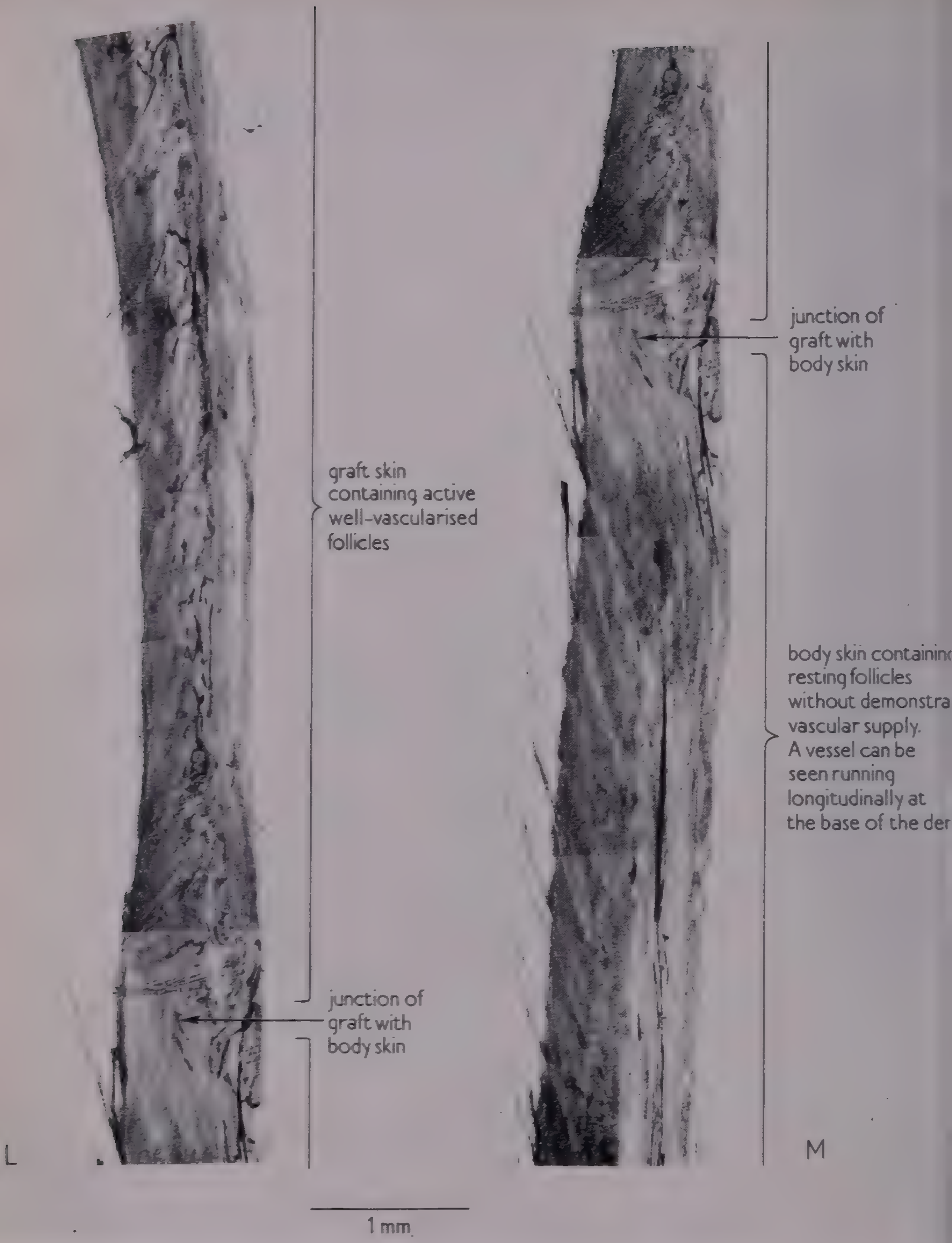
F. J. EBLING and ELIZABETH JOHNSON

Plate 1


graft skin containing resting follicles without demonstrable vascularisation
junction of graft with body skin
body skin containing active well-vascularised follicles
K
1mm

F. J. EBLING *and* E. JOHNSON

Plate 2



F. J. EBLING *and* E. JOHNSON

Plate 3

Immunochemical Studies of Muscle Proteins in Mature and Regenerating Limbs of the Adult Newt. *Triturus viridescens*

by HANS LAUFER[1]

From the Department of Zoology, Cornell University, and the Department of Embryology, Carnegie Institution of Washington

WITH THREE PLATES

INTRODUCTION

QUALITATIVE and quantitative changes which occur in proteins and other macromolecules during differentiation and growth in embryonic development may be detected and defined by immunochemical means (Ebert, 1954, 1959). Similar changes must also occur during regeneration of a part, such as the limb of the salamander, since, after an initial period of cellular breakdown and 'dedifferentiation', regeneration repeats in many ways the embryonic processes. There is the appearance of embryonic-like cells of the blastema which then multiply rapidly and differentiate. In the present investigation immunochemical techniques of studying the changing protein spectrum during development are applied to regeneration in the newt. During the course of this study an analysis of muscle protein regeneration in the larva was published (DeHaan, 1956).

Following initial exploration of the various protein systems, the present study was restricted to the changes in the dedifferentiation and development of the contractile proteins, actomyosin and myosin. In recent years a valuable body of information on the reactions of contractile proteins in myogenesis has been published (Ebert, 1953; Ebert, Tolman, Mun, & Albright, 1955; Holtzer, Marshall, & Finck, 1957). The methods of these workers were employed in part here. In addition, the problem necessitated a re-evaluation of some of the available techniques and their further elaboration. Consequently, a large part of this paper is devoted to technical problems. However, since these serve to improve existing methods, they are presented here in some detail.

Preliminary accounts of this work have been presented to the American Society of Zoologists (Laufer, 1957, 1958) and in the *Carnegie Institution of Washington Yearbook*, **57,** 333–6.

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MATERIALS AND METHODS

Isolation of newt antigens

Tissue homogenates and purified muscle proteins of the newt *Triturus viridescens* were employed as antigens. Tissues were removed by dissection, after which they were extracted in a suitable volume of cold salt solution (0·16 M or 0·6 M NaCl or KCl). All reagents were made up either in glass-distilled water or distilled water passed through a self-indicating mixed bed ion exchange column prepared from Amberlite MB-3. Small quantities of tissue, including the regenerates assayed in this study for muscle proteins, were homogenized in serological test-tubes (6 × 50 mm.) fitted with carborundum-etched plungers. The protein content of most extracts was determined using the Biuret reagent (Gornall, Bardawill, & David, 1949). Protein content was estimated also by the ultra-violet (UV) absorption at 287 mm. on a Beckman DU spectrophotometer.

Isolation of newt actomyosin

Actomyosin was prepared from newts following a modification of the procedure described by Szent-Györgyi (1951) for ‘natural actomyosin’, or ‘myosin B’, of rabbit muscle. Eviscerated, decapitated, and exsanguinated newts were chopped in a meat grinder and then homogenized for 3 minutes in a Waring Blender with 3 volumes of Weber’s solution (0·6 M KCl buffered with carbonate to a pH of 8·5). Extraction was carried out overnight. The crude black extract was centrifuged, after which actomyosin was precipitated from the amber-coloured, viscous, opalescent supernatant by diluting it to a final concentration of 0·05 M KCl with distilled water. Actomyosin was purified by 2 or 3 reprecipitations. Crude actomyosin usually contained a yellow pigment, but reprecipitation yielded a creamy product which could be dissolved almost completely in 0·6 M KCl. The purified actomyosin solution was used in antibody production, in immunological tests, and physical measurements.

Isolation of newt myosin

Purified newt actomyosin was also used for the isolation of myosin. Following the procedure of de Villafranca (1956) for frog myosin, the actomyosin complex was dissociated with 0·1 M $MgCl_2$. Myosin remained in solution during high speed centrifugation. Mg^{++} (which inhibits myosin adenosine triphosphatase) was removed by adding an equivalent gramme molecular weight of the chelating agent, Versene (disodium salt of ethylenediamine tetraacetic acid, EDTA). Myosin was precipitated by lowering the ionic strength, and was further purified by two reprecipitations after dissolving the precipitate in 0·6 M KCl. Traces of Versene were removed between precipitations by dialysing the myosin solution against 0·6 M KCl. Newt myosin was always white. About 2 mg. were obtained from each gramme of newt tissue.

Biochemical and biophysical tests of muscle proteins

In order to determine whether the muscle proteins fulfilled the criteria for contractile proteins established by Szent-Györgyi and others, the following tests were performed on samples of isolated actomyosin and myosin. Adenosine triphosphatase (ATPase) activity was assayed according to the method described by Umbreit, Burris, & Stauffer (1949), phosphorus being determined by the method of Sumner (1944) with a Beckman model DU spectrophotometer. Contractile activity was studied by the addition of ATP and Mg^{++} to artificial fibres formed from the isolated proteins according to the method of Szent-Györgyi (1951). Viscosimetric measurements employed the Ostwald–Cannon–Fenske viscometer, the effect on the viscosity of the proteins of added ATP being investigated. Paper electrophoresis analyses of the proteins were also carried out.

Preparation of antibodies

In preliminary experiments on rabbits, the route and frequency of injection and concentration of antigen were varied extensively. Pre-sensitization, booster shots, and the anamnestic reaction were used to increase antibody titres. Best results were obtained when a series of six injections of antigen was administered intravenously on alternate days, followed by a 4-week rest and then by two or three additional series of injections. The first inoculation of each subsequent series was made subcutaneously to prevent anaphylactic shock. A total of from 30 to 50 mg. of protein was injected in graduated doses to produce precipitating antibodies. An antiserum had a 'working titre' if it precipitated with a 1:512 dilution of antigen originally containing about 0·25 mg. of protein. Thus our antisera could detect 0·5 μg. of protein. Precipitating antibody titres were checked 5 to 9 days after each series of injections, as many as 60 to 70 ml. of blood being taken from the marginal ear-vein of the injected rabbits. The samples were free of contaminating micro-organisms provided aseptic conditions (Boyd, 1956, p. 738) were observed. The sera were collected after centrifuging and 2 to 5 ml. aliquots were frozen and stored at −20° C. in test-tubes until needed.

Immunochemical procedures

Tests of antibodies in vitro

Precipitin and agar diffusion methods were employed. All solutions contained streptomycin, about 1 mg. per ml., and merthiolate, 1/10,000. Muscle-protein solutions were buffered at pH 8·5 in 0·45 M KCl.

A few experiments were carried out using Oudin tubes in which antigen was placed over a column of antiserum-agar (Oudin, 1952). Preliminary exploration revealed the double diffusion technique of Ouchterlony (1948) to be advantageous: when antigen and antibody solutions are placed in separate reservoirs in an agar gel contained in a Petri dish, they diffuse into the agar. Several antigens and antibodies diffusing toward each other in agar produce several lines because each antigen usually has its own rate of migration. Several solutions of antigen can be tested simultaneously against one antiserum (or several antisera can be assayed with one antigen preparation). Confluence of antigen-antibody precipitates from adjacent reservoirs serves as a useful indicator in identification of components (Oudin, 1952).

Small Petri dishes (60 × 15 mm.) were used into which 1·0 per cent. cleared agar was poured in a bottom layer. Six or seven serum bottle caps served as reservoir moulds. These were placed in a circle 5 mm. from a larger central one. A second layer of agar was poured to partly embed the caps. The diameter of the reservoirs after removal of the caps from the gelled agar was 6 to 10 mm. respectively; the depth of the depressions was usually about 2 mm. From 0·04 to 0·08 and 0·08 to 0·13 ml. of test solution was added to the well.

Tests of antibodies in vivo: *infusion of antisera into the regenerate*

Antisera, normal sera, and various control solutions were infused into regenerating newt limbs by the micro-infusion procedure of Singer, Weinberg, & Sidman (1955). The procedure has the advantage of continuously administering minute quantities (about 0·001 ml./hr.) directly into the regenerate. Infusions were performed on newts whose limbs had been amputated about one-third of the length of the upper arm, proximal to the elbow, and then allowed to regenerate to the blastema or early regenerate stage (see stages of Singer, 1952) or to a later stage at which they were infused for 2 to 5 hours. During the infusion animals were anesthetized with 30 per cent. saturated chlorobutanol (Parke, Davis, & Co.). They were observed during infusion, and at intervals thereafter, for pathological signs.

Tests of infusion procedure: infusion of I^{131} labelled gamma globulin

Twelve limbs were infused in the same manner as described in the preparation of unlabelled antiserum, except that the gamma globulin fraction of the serum, labelled with I^{131}, was used. From these limbs 30 autoradiograms were prepared. Infusions were performed for 3 to 4½ hours. The time interval between infusion and fixing was varied from 20 minutes to 9 hours.

In isolating gamma globulin, 25 ml. of fresh rabbit antiserum to newt muscle was prepared according to the ethanol fractionation procedure of Cohn as modified by Goldstein & Anderson (1957). The lyophilized powder was stored in refrigerated evacuated containers with silica gel desiccant. This lyophilized powder, when reconstituted in a buffered bicarbonate-saline solution, was shown to contain precipitating antibodies.

Batches of carrier-free I^{131}, kindly supplied by Dr. M. Berman of the Marine Biological Laboratories, Woods Hole, Mass., and estimated to have an activity of about 100 and 500 microcuries at the start of the labelling procedures, were evaporated to less than 0·5 ml. Iodination was carried out according to the procedure of Talmage, Baker, & Akeson (1954), using 0·3 and 0·4 ml. portions of centrifuged gamma globulin solution containing 4 and 7 mg. of protein respectively. After a few minutes the labelled protein was dialysed against saline overnight at 4° C. with numerous changes of the dialysing medium.

After dialysis, 0·01 ml. of diluted (1:5) newt blood in the first sample, and a mixture of a liver, kidney, spleen, and testis extract in the second, were mixed with 0·2 ml. of the labelled antibody to remove non-specific cross reactions by absorption, following the suggestion of Vaughan & Kabat (1953). A precipitate appeared immediately, showing that the labelled protein had antibody activity even though it was not entirely specific to muscle. Precipitation was continued for about 24 hours with removal of the non-specific precipitate by centrifugation and subsequent addition of trace quantities of absorbing antigens.

Following infusion, the stumps were fixed, decalcified, dehydrated, embedded, and sectioned according to the procedures described below, except that the time in each solution was reduced as much as possible to avoid excessive decrease in potency of the radioactivity of the iodine label. The slides were monitored to estimate the film exposure time. There was sufficient activity in the limbs to produce autoradiograms which made use of Kodak autoradiographic stripping film, placed directly over the slides, as described by Pearse (1953). The sections were stained through the film with acid haematoxylin. Qualitative microscopic observations as well as a number of quantitative grain counts were made using an ocular grid micrometer. Newt limb sections not treated with labelled antiserum had been exposed to stripping film to note any photochemical reducing activity that might be present in the tissues.

Histological techniques

Normal limbs and regenerates, including the infused specimens, were amputated at the base, close to the body, and fixed in a modified Bouin's fluid following the procedures of Singer (1954). The limbs were decalcified for about 48 hours in a chelating agent, 'Decal', after which they were embedded in paraffin wax and sectioned serially at 10 μ. The tissues were stained by the silver proteinate (Protargol) technique of Bodian (1936). Counterstaining was usually accomplished by Orange G. The silver proteinate procedure is a suitable general stain, as well as a special one for nerve fibres.

RESULTS

Normal regeneration of the forelimb of the adult newt

A description of normal limb regeneration in the adult newt is given in various works of Singer (see review, 1952) and only a few points of special interest for the present work need be touched upon here. During the early days after amputation and wound healing there is a considerable destruction of underlying tissues, including partial sarcolysis (Thornton, 1938), in which cut muscle fibres lose their striations near their damaged ends, some nuclei with adhering cytoplasm being freed to become cells of regeneration. These modified muscle-cells, along with other cells now similar in appearance, accumulate under the wound epidermis and form a small mound, called the blastema, on about the 12th day. By this time the muscle of the stump is undergoing its major histological dedifferentiation. Only cut fibres are involved, leaving about one-half of

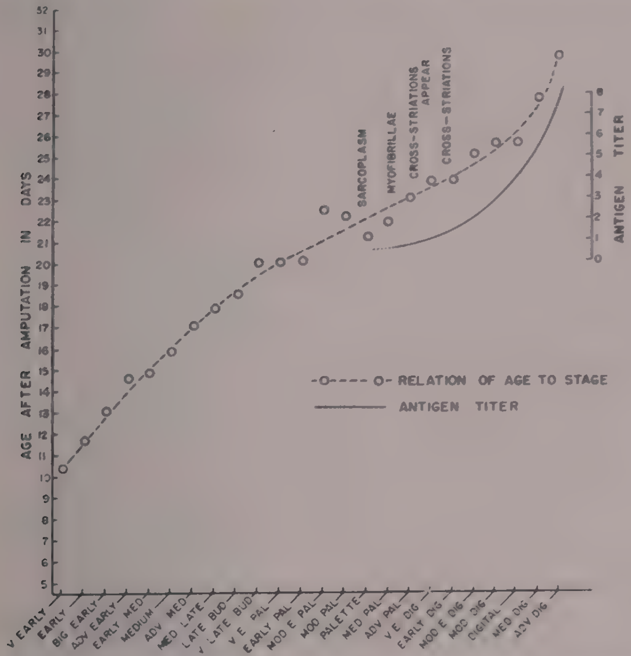
TABLE 1

*The regeneration of muscle in the newt limbs**

Day after amputation	*Signs of dedifferentiation*	*Regeneration in proximal stump*	*Regeneration in regenerate*	*Stage*
10	+	none	none	v. early bud
13	+	slight hint of start	none	early bud
14	trace or end	start, mitoses in nuclei of future muscle mass	none	adv. early bud
15	—	early regen. of fine fibres along side of bone	none	medium bud
16	occasional muscle fibre breaking down	extensive fine fibre regen.	earliest striated fibres extend just past level of bone in stump	medium late bud
18	—	extensive regen.	extension of myoblasts to regenerate is marked; no cross striae as yet beyond level just above bone	very late bud
21	—	+	stump muscle beyond level of bone extending with cross striae. Occasionally *de novo* muscle with few fine cross striae, but not as yet beyond old epidermis. Myoblasts well distributed distally	palette
23	—	+	young muscle cells extending well into regenerate; occasional cross striated fibres. New muscle cells aggregated into chains, some with thin striations	early digital
24	—	+	young muscle cells extending well into regenerate, with cross striations beyond old epidermis	early digital
54	—	+	extensive muscle growth	adv. digital

* Most slides from collection (series EL and DL) of Prof. Singer. At days 23 and 24 several additional normal specimens were used.

the muscle of the stump intact. The place of the sarcolysed muscle is taken by various mesenchymatous elements, presumably of muscle and connective-tissue origin. There is then rapid cellular proliferation and major increase in size of the regenerate during the next week until it becomes dome-shaped and, still later, flattened at its end to form a hand from which digits will later differentiate (Singer, 1952). With morphological differentiation there is also histogenesis, first of cartilage, then of muscle and connective tissue. The time when muscle is first formed is recorded in Table 1 and Text-fig. 1. In evaluating the experimental results further reference will be made to the time-course of these developmental events.



Text-fig. 1. Comparison of average normal regeneration rate with the appearance of muscle antigen and histological differentiation of muscle.

The transition between sarcolysis and differentiation of new muscle fibres is very subtle. Muscle fibres in one part of a section may be undergoing sarcolysis while other fibres appear to be forming new extensions at their ends. In the muscles of the stump which underwent partial sarcolysis after cutting, the breakdown ceases and the remaining fibres begin to regrow toward the blastema by a process called 'cytoplasmic budding' by Thornton (1938). Along with such budding in the stump there appears to be muscle formation from individual cells (presumably myoblasts) in the distal regenerate; these cells group together to form aggregates which later form a continuous syncytium. Myofibrillae and cross-striations soon appear.

The partly histolysed muscle of the stump is the first site of muscle proliferation. It has started growing by the time the palette is formed. Fibres of different stages of maturation can be seen next to one another at the same level. The regenerating fibres then gradually extend into the regenerate. Differentiation of striations usually occurs several days after the appearance of the fibre. Later, at about the late palette or early digital stages, myoblasts destined to form distal muscles appear within the regenerate itself. Fibrillae differentiate early and are seen in bundles around the myoblasts. Later still, development includes further elaboration of the fibres, their increase in number, and organization into discrete muscles.

Characteristics of newt actomyosin and myosin

The relative viscosity of a purified actomyosin preparation estimated to contain 2 mg. of adult normal protein per ml. of Weber's solution was 1·44 at 4° C., a value which is in good agreement with those reported by Szent-Györgyi for rabbit actomyosin. The viscosity of the preparation dropped noticeably upon the addition of ATP. Finally, actomyosin preparations were shown to have ATPase activity. The actomyosin migrated as one component in paper electrophoresis. All of the findings are consistent with the view that the use of the term 'actomyosin' for our preparations is fully appropriate.

Tests performed on myosin preparations showed that they differed markedly from actomyosin. The intrinsic viscosity was unchanged by the addition of ATP. The preparations were capable of forming artificial fibres which did not contract on the addition of ATP. However, ATPase activity was present. Electrophoresis on paper did not result in further resolution. All the tests support the conclusion that the material isolated was myosin.

Reactivity and specificity of antibodies

The injection of various newt antigens into rabbits resulted in the production of antibodies capable of forming visible precipitates which, in agar diffusion tests, varied from faint to sharp lines. Eight out of twenty rabbits injected produced antisera which contained precipitating antibodies capable of forming one or more sharp bands. Weak antisera were usually produced by 0·16 M NaCl extracts of whole newts. With repeated injections of antigen, working titres of antibodies were obtained. Interestingly, all the mixtures which contained muscle proteins, whether whole newt extracts, homogenates of dissected muscle, actomyosin or myosin preparations, evoked the same antibodies in at least some of the animals. This result is illustrated in Plate 1, figs. 1–4. The anti-myosin formed in rabbit 6 in response to the injection of whole newt extract (Plate 1, fig. 2, wells B, C) is similar to the anti-myosin formed by rabbit 23 (Plate 1, fig. 1, reservoir C) upon injection of purified myosin. The similarity in the response of several rabbits to the injection of different types of muscle antigens indicates that the extensive purification of myosin did not alter its immunochemical properties. In our best preparations there was a characteristic major component ror actomyosin and a different major component for myosin.

Factors influencing agar diffusion

Sera from normal rabbits did not react with newt antigens. Streptomycin and merthiolate in twice the concentrations customarily used for preserving protein solutions did not visibly effect the formation of a precipitate.

The potency of each antiserum was first measured according to its ability to react with serially diluted solutions of antigen in ring precipitin tests. When antigens of adult tissue extracts were added to antisera incorporated in agar in Oudin tubes, a reaction was observed at the interface, but the antigen-antibody complex failed to penetrate into the agar except on rare occasions, despite good precipitin reactions in ring tests. Experiments indicated that the following variables were not limiting agar penetration, though they may influence the precipitin reaction to some extent: pH between 6·0 and 10·0, salt concentration between 0·16 M and 0·6 M, agar concentration, and several types of buffering systems. Nor did the use of starch, gelatin, or silica gel improve the results. The observation was then made that nearly all of the diffusion occurred in the first few hours, when it was occasionally observed in tubes maintained at 25° C. This forcefully demonstrated the lability of the antigen. When Oudin tubes were kept at 4° C., successful diffusion into agar was obtained. Thereafter, only fresh preparations (less than 3 days after extraction) were used in agar diffusion studies conducted at low temperatures.

Maintaining Oudin tubes or Ouchterlony plates in the refrigerator permitted diffusion to occur; but with frequent removal for observation several additional bands appeared. The number of bands seemed to be correlated with the frequency of observations at room temperature, thus implicating the induction of temperature artifacts by rapid and large temperature changes (compare Plate 1, figs. 1, 5–7). Such temperature artifacts have been reported several times for Oudin tubes (Oudin, 1952), and, more recently, by Munoz (1954) for agar plates. Maintaining plates in a cold room lowered the frequency with which these additional striae appeared, and the diffusion of bands in agar was also improved. More recent plates show sharp bands well defined and clearly separated when not antigenically related. These results have subsequently been repeated and reconfirmed a number of times to eliminate the possibility of artifact.

Identification of muscle antigen-antibody complexes precipitated in agar diffusion plates

When anti-actomyosin sera were tested separately against extracts of newt brain, skin, blood, liver, testis, egg, spleen, and heart, they were found to cross-react with all of these tissues. Complete absorption with any of the non-muscular tissue extracts eliminated further reactivity with any of the heterologous tissue. A specific reaction for muscle extracts remained, indicating that at least one component of the antisera was not organ-specific; furthermore, it reacted with a common constituent probably contained in all the body tissues.

When an antiserum to newt actomyosin was allowed to react with a 0·6 M saline extract of whole fresh newt in agar, a minimum of three bands was observed which repeated tests proved not to be artifacts (see Plate 1, figs. 1–5, well A). Two of these bands were similar in their migratory characteristics, and, in fact, could not always be resolved because they migrated closely together (see, for example, Plate 1, fig. 7, wells A, D; fig. 8, wells A, B; also Laufer, 1957). They were resolved into a heavy, slowly migrating band and a lighter, faster minor component in a number of experiments (see, for example, Plate 1, figs. 1–6). Their penetration was relatively slow and extremely temperature-sensitive. Temperature changes resulting from handling produced several striae. Moreover, there was little or no penetration into the agar if the antigen solution was aged or disturbed.

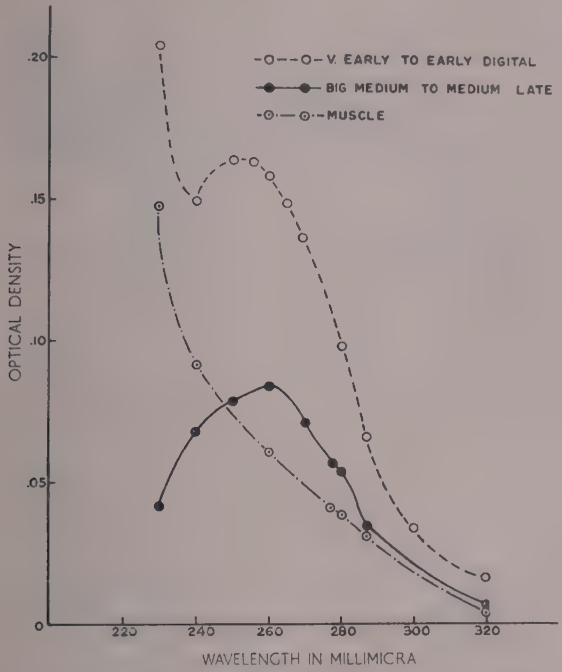
TABLE 2

Antibody-antigen reactions in agar: anti-actomyosin and adult newt antigens

Antigen	Antiserum	*Positive reactions/no. of tests*	
		Non-specific precipitates: rapid components (*nucleoprotein*)	*Muscle precipitates: slow components*
Actomyosin (purified)	Anti-actomyosin	0/4	4/4
Myosin (purified)	,,	0/4	4/4
Frog myosin (purified)	,,	0/4	4/4
Muscle extract	,,	23/23	23/23
Whole newt extract	,,	42/42	42/42
Organ extracts (brain, blood, liver, spleen, testis, ovary)	,,	23/23	0/23
Heart extract	,,	6/6	5/6

The third component migrated readily to form a band of precipitate closer to the antiserum reservoir than to the other components. The band varied in sharpness and was not usually as heavy as the major band described earlier (Plate 1, figs. 1–7; Plate 2, figs. 8–12). It, too, was absent in aged antigen solutions.

When the antiserum, which had one major (the slowest) and two minor (more rapidly migrating) components, was tested with extracts of muscle, skin, spleen, ovary, heart, testis, oviduct, brain, egg, liver, as well as with actomyosin and myosin, all tissues showed at least one antigen in common, namely, the rapid component (see Plate 1, figs. 5–7; Plate 2, figs. 8–10; Table 2). The band could be removed or absorbed first by causing the antiserum to react with any non-muscular newt tissue, e.g. blood. When this was done, only muscle protein antibodies were left in solution. Only the slower band or bands remained when the blood-absorbed antiserum reacted with muscle extracts in agar diffusion. The bands of precipitate developed with agar diffusion agree fully with the results obtained with conventional precipitin techniques demonstrating at least one general tissue antigen and a minimum of two specific muscle components (see Table 2; Plate 1, fig. 1; Plate 2, figs. 8, 9). The general tissue antigen was present in all *Triturus* tissues tested, but absent from the frog, and thus exhibits species specificity.



TEXT-FIG. 2. Typical absorption curves of several antigen extracts (curves adjusted for 1 mg. wet weight of tissue/ml. of 0·6 M KCl, 0·1 N NaOH).

Since the method of preparation makes it inevitable that actomyosin would be contaminated with nucleoprotein (for this class of molecule is also purified by dissolving at high ionic strength and precipitating at lowered salt concentration), a good clue was provided to the nature of the third antigen. The lability, basophilic staining, the characteristic absorption at 260 mμ (Text-fig. 2), and the partial digestibility of antigen preparations with ribonuclease indicated that nucleoprotein was present. Other possible contaminants such as myoglobin and serum proteins (Cooper, 1946, 1948, 1950) were ruled out.

It is suggested, therefore, that the non-specific (rapid) component was probably a nucleoprotein, while the evidence indicates that the slower major band

TABLE 3

Antibody-antigen reactions in agar: antigens of regenerating newt limbs

							Positive reaction/number of tests completed	
Stage tested	*Age in days*	*Number of specimens tested*	*Average wet wt. in mg.*	*Average protein mg./ml.*	*Average volume used per reaction*	*Test antiserum*	*Non-specific precipitate*	*Muscle precipitate*
Big early bud . . .	17	7	0·26	19	0·04	actomyosin	2/2	0/2
Big medium bud . . .	17	10	0·64	31	0·04	actomyosin	5/5	0/5
Medium late bud . . .	17	3	0·6	18	0·04	actomyosin	2/2	0/2
Late bud	20	18	0·65	38	0·04	actomyosin (myosin)	7/7 (0/2)	0/7 (0/2)
Early palette	21	12	0·89	31	0·04	actomyosin (myosin)	2/2 (0/2)	1/2 (0/2)
Moderate palette . . .	17*	13	0·97	90	0·05	actomyosin (myosin)	2/2 (0/1)	0/2 (0/1)
Palette	25	21	0·95	55	0·04	actomyosin (myosin)	5/5 (0/3)	4/5 (2/3)
Very early digital . . .	21 & 17*	17	1·18	53	0·045	actomyosin (myosin)	4/4 (0/2)	1/4 (2/2)†
Early digital	26	33	1·6	57	0·04	actomyosin (myosin)	8/8 (0/4)	6/8 (4/4)†
Moderate digital . . .	33	40	2·8	133	0·04	actomyosin (myosin)	13/13 (0/7)	13/13 (7/7)†
Medium digital . . .	38	15	2·6	89	0·04	actomyosin (myosin)	3/3 (0/4)	3/3 (4/4)†

* Reamputated limbs have been used; these regenerate somewhat more rapidly than usual.

† Absorption procedures have been used with a number of these reactions.

was probably actomyosin, and the third component myosin. Anti-actomyosin and anti-muscle-homogenate sera react to form the same types of bands (muscle and nucleoprotein), although these sera vary in the intensity of their precipitating ability. Purified myosin evoked highly effective anti-myosin sera, some of which showed only one band when tested with muscle extracts. In some rabbits, anti-actomyosin antibody was formed along with anti-myosin in response to myosin preparations (see, for example, Plate 1, fig. 1, and compare reservoirs A, B, and C).

The appearance of muscle antigens during limb regeneration

Agar diffusion tests with anti-actomyosin sera

Extracts of regenerates in various stages of development were prepared in Weber's solution and allowed to react with anti-actomyosin sera in Ouchterlony plates. Each plate also contained one reservoir of crude muscle extract as a positive control. In 71 separate tests of regenerate and control tissues all regenerates reacted to form the non-specific nucleoprotein band, but only regenerates in the palette, digital, and later stages gave reactions specific to muscle proteins. Extracts of regenerates in various digital stages consistently revealed actomyosin. Extracts from early digital and palette stages usually showed only a small quantity of actomyosin, and occasionally none could be detected. It was not possible to demonstrate actomyosin earlier than in the palette stage (cf. Table 3; Plate 1, fig. 7; Plate 2, figs. 10–12).

Agar diffusion tests with anti-myosin sera

In 25 tests using anti-myosin sera on extracts of the same limbs used in tests for actomyosin, and in additional control tests simultaneously performed, completely consistent results were obtained. The non-specific component was not demonstrable with these antisera. Myosin could be detected in the palette stage and thereafter, but not before. By the early digital stage the myosin component was present, in a number of tests, in sufficient quantity to penetrate substantial distances into the agar. When the antiserum contained antibodies to both actomyosin and myosin, two distinct bands could be seen at this stage (Table 3; Plate 2, figs. 12, 13).

Infusion of normal sera and antisera

Infusion of normal rabbit serum, or serum absorbed with newt blood, into regenerating newt limbs produced a general cytotoxic reaction. The extent of the reaction varied among individuals and was influenced at least in part by the amount of serum infused. The reaction is termed 'general' because all tissues appeared to be affected equally as determined by macroscopic and microscopic examination. Neither was there any specificity according to the age of the regenerate.

TABLE 4

The effect of infusing anti-muscle serum and control reagents upon the structure and rate of regeneration of the newt limb

	Experimental effect	*Number of limbs observed*	*No effect*	*Number of days limbs were affected*								*Number affected*	*Per cent. affected*	*Average number of days affected*
				1	*2*	*3*	*4*	*5*	*6*	*7*	*7+*			
Control	Saline infused 4 hours:													
	Inflammation	19	16	2	1							3	16	0·21
	Delay	18	16	1		1						2	11	0·22
I. A	Serum obtained before antibody formation, absorbed,* infused 3 hours:													
	Inflammation	45	10	8	12	7	3	3	1			35	78	2·0
	Delay	44	27	3	8	1	2					17	39	1·3
B	Serum with anti-actomyosin antibodies, absorbed,* infused 3 hours:													
	Inflammation	28	0	3	2	6	8	2	2	4	1	28	100	4·1
	Delay	27	1	7	7	3	6	1				26	96	2·8
II. A	Serum before antibody formation, absorbed,* infused 3 hours:													
	Inflammation	17	1	7	2	4	3					16	94	2·0
	Delay	17	10	3		1						7	41	0·8
B	Serum with antimyosin antibodies, absorbed,* infused 3 hours:													
	Inflammation	18	0	2	2	4	9		1			18	100	3·3
	Delay	14	1	4	1	3	3	1	1			13	93	2·5
III. A	Antimuscle serum diluted 1:1 with saline, absorbed,† infused 4 hours:													
	Inflammation	8	0	3	3	2						8	100	1·9
	Delay	7	4	0	2	1						3	43	1·0
B	Antimuscle serum diluted 1:1 with saline, absorbed,* infused 4 hours:													
	Inflammation	52	7	5	9	13	10	4	2			45	87	2·8
	Delay	47	13	6	6	6	5	4	5	2		34	72	2·6

* Absorbed with newt blood until non-specific cross-reactions were eliminated.

† Absorbed with muscle antigen.

The cytotoxic reaction included the formation of blisters. At times rather extensive ones were produced (Plate 3, fig. 14). Internal bleeding, hyperaemia, and sloughing of tissues was also apparent (Plate 3, fig. 15). The destructive phenomena persisted for from 1 to 8 days, accompanied by a delay in the rate of regeneration (Table 4; Plate 3, figs. 14–19).

Histological examination revealed general cellular destruction, pycnotic nuclei, phagocytes, and scattered pigment granules in the epidermis and mesenchyme-like regions.

When antiserum, previously absorbed to remove any non-specific activity, was infused into regenerates of various stages, the same general cytotoxic effects and inflammatory reactions were noted as when normal rabbit serum was introduced into the regenerate. However, the effect occurred in a greater number of animals and the changes observed persisted for a significantly longer period (see Table 4). An anti-actomyosin serum doubled the average extent of the cytotoxic effects and the delay period (experiments 1A and 1B). Similar results were observed with anti-myosin serum and anti-muscle serum (experiments II A and B; III A and B).

Histologically, in addition to causing the effects noted for normal serum, antiserum also affected muscle fibres in another way. These sera caused vacuolation in old as well as in newly-forming and young muscle fibres between the 1st and 2nd days after infusion. Next, the fibres began to disintegrate with the formation of a considerable number of large and small granules of basophilic material and sarcoplasmic particles of various sizes. Phagocytic cells were common among disintegrating muscle fibres. Examination of more than 170 sectioned limbs indicated that degeneration of muscle fibres was restricted to limbs infused with muscle antibody. Muscle fibres were thus affected by their specific antisera, and in a number of limbs this was the only effect. Muscle degeneration due to antiserum infusion is compared with normal dedifferentiation in Plate 3, figs. 20–22.

Limbs infused with antiserum or control serum, and un-infused limbs, could be distinguished by the investigator on coded slides with an accuracy of about 80 per cent.

After several days of muscle disintegration the damaged stumps and regenerates initiated repairs and eventually all resumed regeneration. However, these limbs lagged considerably in their rate of muscle growth.

Experiments performed to gain further insight into the non-specific toxicity of rabbit serum indicated that the non-dialysable fraction was responsible. Other foreign proteins, such as purified beef haemoglobin, also had similar effects, while homologous protein such as newt haemoglobin did not produce signs of toxicity. We considered these results to indicate that the toxic manifestations are attributable to as yet little understood foreign protein reactions.

Infusion of labelled gamma globulin into the regenerate

Radioactivity gave visual evidence of the infused material, the fate of which could be followed several hours after infusion. The knowledge obtained from the movements of material in experiments with labelled gamma globulin served to indicate the course of similar but unlabelled proteins reported in the foregoing section.

Results immediately after infusion showed that the infusate permeated all regions of the regenerate and a considerable part of the stump. The protein moved mostly within intercellular spaces (see Plate 3, fig. 23), but some entered the blood-stream. Substantial concentrations of labelled material reached the proximal adult tissues of the stump as time passed. A few hours after infusion the amount of labelled globulin in the blastemal area became less marked. The skin lost its high concentration of radioactivity. Grain counts performed on specimens fixed from 5 to 9 hours after infusion showed that there was a concentration of globulin, especially in the adult muscle of the stump; bone, cartilage, and skin were relatively low in activity (Plate 3, fig. 24; Table 5).

TABLE 5

An example of total grain counts of an autoradiogram taken at four regions along the axis of a cross-section of a limb after infusion of I^{131}-labelled antiserum

Level of section	*Tissue counted*	*Total grain counts* (in 12 squares)*
1	Bone	36
	Epidermis	87
	Muscle	272
2	Bone	17
	Epidermis	56
	Muscle	149
	Nerve	39
3	Bone	16
	Epidermis	35
	Muscle	122
	Nerve	45
4	Bone	10
	Epidermis	26
	Muscle	159
Background		10

* The grain counts of these categories were used in an analysis of variance. Statistically significant differences between the antibody localization of the different tissues was demonstrated.

The blastema eliminated a large part of the foreign material; in this elimination the skin apparently played an important role, as seen by the leakage of radioactive material from the surface of the skin of the blastema (Plate 3, fig. 24). The higher grain counts for skin over and above those for some tissues nearer the infusion site further suggests an active pick-up and transport of foreign material out of the limb through the skin.

That some of the labelled gamma globulin is eliminated from the limb via the blood-stream is clear not only from general considerations, but also from observations in the specimens. Further substantiation of the importance of this mode of elimination of foreign matter from the limb (and also for the localization of antibodies) is found in those limbs in which the circulation was noted to be poor previous to fixation. In these, a great abundance of labelled material floods the entire limb-tip with relatively little diffusion or localization. Most of the activity is in the regenerate and in the tissue spaces of the limb-stump.

DISCUSSION

Among the important technical questions raised in this investigation are the following: how successful are the procedures for isolation and characterization of actomyosin and myosin of the newt? What are the problems involved in production and assay of antibodies for newt proteins? Especially pertinent are questions raised by the use of agar diffusion methods. How may we evaluate the cytotoxic effects of muscle antisera on the regenerating limb following the infusion of small quantities of sera? Finally, brief comment on the use of radioactively labelled gamma globulin is warranted.

Applying the criteria established by Szent-Györgyi for rabbit actomyosin, it was shown that actomyosin extracted from the newt was relatively pure, capable of contraction, and fulfilled other standards as well. Myosin, too, when isolated from the newt, met the standard biochemical and biophysical criteria described by others.

Antigenicity of muscle proteins

Both myosin and actomyosin of the newt are antigenic in the rabbit. It cannot be said, however, that they are good antigens, for their lability and solubility properties make them difficult to handle.

The problems encountered in producing antibodies to myosin have already been described by Ebert (1953; Ebert *et al.*, 1955). Furthermore, there are indications that antibodies to muscle proteins have a broad spectrum of cross-reactivity with the muscle proteins of other species. For example, Holtzer's myosin (Ebert, 1959) also reacted with an antigen in salamander muscle. Moreover, the injection of tissue extracts, e.g. of brain, into animals of the same species may result in the production of auto-antibodies capable of reacting with and destroying the antigen *in situ* (Boyd, 1956). Antibodies of muscle proteins produced in rabbits may not produce lesions, as do anti-brain sera; however, it is also possible lesions might go undetected because of the greater volume of muscle. It may be that antibodies to newt muscle react with, and are absorbed by, rabbit muscle. Such a combination of antibodies and antigens *in situ* might explain the need for early bleeding of rabbits to obtain antibodies after immunization. The unusually rapid drop in the titre of myosin antibody described by Ebert (1953) and noted in the foregoing section can be accounted for in this way.

The poor antigenicity of newt muscle protein in rabbits may also be attributed to the high lability of the antigen. In addition, immediately upon injection into the rabbit the salt concentration of the antigen is lowered, rendering most of the antigen insoluble and possibly eliminating a substantial fraction of the antigen solution. These factors doubtless contribute to the difficulty of obtaining high titres of anti-muscle sera.

Antibodies to actomyosin, myosin, and a third fraction, believed to be nucleoprotein, were obtained. These three antibodies were consistently revealed by agar diffusion analysis. Injections of saline-extracted whole newts produced these antibodies in some rabbits. It may be concluded, therefore, that in spite of the difficulty in obtaining antibodies to newt proteins, actomyosin, myosin, and nucleoprotein are among the more antigenic compounds present. Agar-diffusion procedures are commonly performed using excess quantities of antigen and antibody to assure a continuous 'infinite' gradient of both reagents for diffusion. However, in the present studies the supply of protein antigens from the regenerate was limited; hence the conventional Ouchterlony technique was modified for small samples. Although the quantity of reagents used was small, the diffusion patterns were consistent and reproducible. The reliability of the modification was established by comparing the results with those observed in tests with an excess of interactants.

Evaluation of the agar-diffusion method

The agar-diffusion method also provides a means of evaluating isolation procedures. There does not appear to be any procedure for the reliable separation of actomyosin without myosin being present as a contaminant (Szent-Györgyi, 1951; Hamoir, 1955; Hamoir & Reuter, 1956). The more rapid the extraction, the more myosin is obtained in the actomyosin fraction. Newt muscle, like that of the frog, is somewhat anomalous in its behaviour during extraction (Hamoir & Reuter, 1956; de Villafranca, 1956) in that myosin cannot be extracted in the conventional manner. As de Villafranca stated, 'methods normally used for preparation of myosin from rabbit muscle always resulted in actomyosin' in the frog. Using de Villafranca's modification it was possible to isolate myosin which, with the exception of traces of actomyosin in some samples, yielded an immunochemically homogeneous product when tested by agar-diffusion. The anti-myosin of such a fraction was always confluent with the more rapidly diffusing (myosin) components of actomyosin preparations in Ouchterlony plates. In the light of the results of de Villafranca and Hamoir & Reuter on frog muscle, and the extension of these findings to immunochemical studies of newts, the ontogenetic studies of myosin (Ranzi & Citterio, 1955) in the frog embryo should be interpreted cautiously. Though referring to their muscle protein as myosin, Ranzi & Citterio were most likely working with a mixture of both actomyosin and myosin.

Many of the muscle preparations were contaminated with nucleoprotein.

Ranzi & Citterio (1955) also recognized the presence of nucleoprotein in extracts of frog muscle and attributed all of the absorption at 260 mμ of extracts of yolk-plug stage embryos to deoxyribose nucleoprotein. The present experiments indicate the presence of ribose nucleoprotein as well.

The possibility exists that not all of the absorption at 260 mμ is due to nucleoprotein or nucleic acids, but to smaller fragments, nucleotides and nucleosides. The present findings with dialysed preparations indicate that most of the absorption at 260 mμ is retained, and is therefore attributable to macromolecules.

Nucleoproteins can elicit the formation of antibodies, although probably not as readily as proteins and certain carbohydrates. Their antigenicity has only recently been established (Blix, Iland, & Stacy, 1954; Phillips, Braun, & Plescia, 1958; Lawlis, 1958).

The present findings suggest that there is more nucleoprotein present in actively growing and proliferating regenerates than there is in mature and non-regenerating tissues. Both ultra-violet absorption studies and comparisons of bands in agar diffusion tests support this view.

It has not yet been possible, however, to prove a direct relationship between the nucleoprotein fraction and the band revealed by agar diffusion studies. In order to demonstrate such a relationship, characteristic staining and specific enzyme digestion must be employed. Only indirect evidence has been presented.

RNAase and DNAase digestion of antigen preparations followed by diffusion failed to prove the identity of the non-specific nucleoprotein band. Calculations based on data presented by Preer (1956) indicated that virtually complete digestion of nucleoprotein would be required before an appreciable difference in agar diffusion could be expected. Niu (1958) stated that he was never able to get more than 71 per cent. RNA digestion with RNAase. A further possible difficulty in observing direct relationships between nucleoprotein and agar diffusion is that DNAase and RNAase digestions probably leave the protein moiety intact, and protein might form the precipitate band in agar diffusion.

An extension of the immunochemical methods may contribute to further our understanding of the structural organization of muscle.

Synthesis of actomyosin and myosin in the regenerating limb

Muscle regenerated in a continuous wave beginning in the stump, and thence distally, with the formation of new fibres in the regenerate itself. Many of the latter fibres arose from newly organized muscle-forming centres. There was considerable variation among individuals, but less between limbs of the same individual in the time at which the events of muscle formation occurred. The regenerating striated muscle fibres of the stump advance to the original amputation level by the late bud stage; muscle-cells with cytoplasm extended into the regenerate by the palette stage. An occasional myofibril appearing in the regenerate in the late palette and very early digital stages preceded the appearance of muscle striae by as much as 2 days.

Actomyosin and myosin were detected in the moderate and early digital stages. When tested with antisera, extracts from earlier stages yielded fainter bands which penetrated the agar to a lesser extent. Small amounts of muscle protein were detected in extracts of very early digitals, and occasionally were detected even in the palette stages. Since extracts were made from regenerates taken distal to the original amputation line, old stump muscle and the early striated muscle growing distally into the regenerate from the stump were avoided. It may be concluded that the immunological reactivity to specific muscle antibodies was detected before cross-striations (as observed by light microscopy of stained preparations) have appeared in the muscle of the regenerate. Admittedly, the reactions of antigen from palette and early digital stages were weak, but they were distinct. These antigens cannot be conclusively identified as adult actomyosin or myosin. It is likely, however, that this was their identity, for immediately after their appearance there is a rapid increase in production of proteins which have the same precipitation and diffusion properties as those characterized as adult myosin and actomyosin. These results agree with those of DeHaan (1956), who performed ring precipitin tests for the appearance of muscle protein in regenerating hind limbs of larval *Amblystoma mexicanum*. He found that muscle proteins were present in easily detectable amounts in a stage equivalent to our 'advanced digital' stage (day 34; cf. DeHaan, 1956, p. 78, figs. 1*h*, 4), and in lesser amounts at 29 and 32 days after amputation ('moderate' and 'medium digital' stages). Thus, specific muscle antigens appear later in larval *A. mexicanum* regenerates than in adult *Triturus* regenerates. One might think that the antigen of the larval limb would develop sooner in relation to the stage of regeneration, or at least no later, because larval regeneration proceeds at a greater rate and is accomplished with more ease than in the adult. Apparently, however, muscle appears relatively late in these larvae (see DeHaan's photomicrographs). The muscle of the freshly amputated larval stump undergoes more widespread sarcolysis than that of the adult. In *A. punctatum* larvae the sarcolytic sweep extends to the shoulder (Thornton, 1938), but in adult *Triturus* sectioned muscle fibres undergo sarcolysis only for a short distance proximal to the cut. Therefore, in subsequent regeneration the adult muscle fibres can be completed sooner than the totally sarcolysed ones of the larva. A second reason for the difference in the stage at which muscle first differentiates may be the size and volume of the regenerate itself. Although the small *A. punctatum* larvae generally regenerate quite rapidly (Thornton, 1938), the hind foot of a 12–13 cm. axolotl (*A. mexicanum*) is a considerable growth compared to the forearm of *Triturus*, and it may take more time to accomplish regeneration of muscle in a large larval limb than with a small growth in an adult form.

The time of synthesis of specific muscle proteins

The time at which specific muscle proteins can first be detected during myogenesis appears to vary from animal to animal and from tissue to tissue. For example, using precipitin reactions Ebert (1953) found cardiac myosin (or 'similar combining groups') present in the mid-streak (stage 3 of Hamburger & Hamilton) of the chick embryo, and Ranzi & Citterio (1955) detected myosin in a similar stage during frog development of the yolk plug (stage 12 of Harrison).

Hibbs (1956) examined myoblast development in the chick heart with the electron microscope and found that 'it is at this stage (30 hours) that the first contractions occur (Patten & Kramer, 1933) although no structures which in any way resemble myofibrils can be seen. . . . The formation of the A band is a more gradual process, which begins only after the myofilaments have assumed their final arrangement.' Hibbs's statement is of unusual interest inasmuch as myosin is commonly believed to be localized in the A band (Hasselbach, 1953; Hanson & Huxley, 1955).

Lewis's (1919) observations with the light microscope on the early chick heart are at variance with those of Hibbs, and they have recently been substantiated for skeletal muscle by Holtzer & Abbott (1958). Lewis found occasional cross-striated muscle fibres as early as in the 30-hour embryo. According to Holtzer, the occurrence of striations may coincide with the onset of contractility.

Holtzer *et al.* (1957) studied skeletal myogenesis in chick embryos employing anti-myosin antibodies tagged with fluorescein isocyanate; their results support the view that myosin is restricted to the A band. They also maintain that myosin is detectable only in cross-striated myofibrils. They report that fluorescent antibodies visualize the cross-striations before they can be seen with the ordinary light microscope or even with the phase microscope, and insist that when myosin is first detected it is confined to the A band (Holtzer *et al.*, 1957; Holtzer & Abbott, 1958), although their own figures (Holtzer *et al.*, 1957, figs. 2, 3) show an unsegmented section of a young muscle-cell which is fluorescent.

The inability to detect actomyosin or myosin in regenerating stages earlier than the palette is of uncommon interest since it is believed by many workers in the field that at least some blastema cells originate from dedifferentiated muscle-cells. If this is so, and there are indications that it is, then the question of the extent of chemical dedifferentiation is raised. The immunochemical data indicate that the loss of muscle proteins during early regeneration may be considerable. Thus, not only extensive morphological but also biochemical dedifferentiation occurs.

Infusion of the regenerate with antibodies

The use of antisera on living cells provides a sensitive method for analysing the development of specific molecules. When anti-muscle sera were infused into the blastema, the destruction took various forms and degrees of severity, but in general was expressed as an interruption and delay in regeneration, after which the latter process was resumed. Even in the face of adverse chemical agents the regenerate is capable of quick recovery. Singer *et al.* (1955) conclude that the process of regulation 'is an inexorable one, thoroughly ingrained into the physiology of the animal and capable of drawing without reservation upon the vital stores of the body to continue or resume growth under most unfavourable circumstances'. Of great interest was the fact that muscle tissue was the principal target of muscle antisera, the effect being a specific cytotoxic activity of the antiserum. Antisera did not affect all muscle fibres equally. Some were demolished, many showed only limited destruction, and others appeared untouched. The major factor responsible for the difference in sensitivity may have been the degree of contact with antiserum. This explanation is supported by the fact that young fibres which were scattered and relatively exposed were a more likely target for attack by antiserum. It may be that young developing fibres are more vulnerable. The reaction was complicated by a general reaction which appears to be a typical response to the injection of foreign proteins. Immune responses of amphibians and other lower vertebrates to foreign protein are well known (Favour, 1958). Adult newts do not readily retain foreign skin grafts, or other implants (see, for example, Horowitz, 1937). The frog also can elaborate antibodies (Bisset, 1947). General cytotoxicity upon exposure of a developing system to antiserum has been reported frequently (reviewed by Nace, 1955, and Mun, 1958).

The course of labelled gamma globulin in the regenerate

The problem of exposure of tissue to infusion medium was evaluated by the use of labelled gamma globulin. Heretofore the infusion technique of Singer *et al.* (1955) had not been tested with protein solutions. Apparently all tissues of the regenerate and distal stump were accessible to protein, though it is recognized that not all cells are equally exposed.

It is difficult to conclude from these experiments whether specific localization due to antisera occurred since the distribution of normal gamma globulin is not known, but the data are suggestive of localization of the muscle antisera about muscle-cells. This interpretation is also consistent with the infusion of unlabelled rabbit serum and antiserum.

Experiments using fluorescent antibodies also bear out this interpretation. Normal rabbit gamma globulin and our anti-actomyosin gamma globulin, in which the two specific muscle protein bands were demonstrable in agar diffusion, were labelled with fluorescein isocyanate. These experiments were performed by Professor J. Gall of the University of Minnesota. The antibodies in this case localized specifically in periodic banding, presumably in the A bands of adult salamander muscle fibres.

SUMMARY

1. Immunochemical procedures were employed to analyse changes in contractile proteins during limb regeneration in the adult newt, *Triturus*. Methods for the isolation of myosin and actomyosin were modified for use in the newt.

After these proteins were characterized biochemically they were shown to be antigenic in the rabbit.

2. The activity and specificity of anti-actomyosin and anti-myosin sera were determined by means of agar diffusion techniques. Purified actomyosin was found to be contaminated by myosin and a second component, a nucleoprotein. The diffusion coefficients increased in the order actomyosin, myosin, nucleoprotein. The injection of myosin into rabbits elicited the formation of antibodies which, when tested in agar diffusion, usually demonstrated a minimum of one band but occasionally, when contaminated by minor quantities of actomyosin, two bands were seen. Some modifications of existing procedures are described for the use of small reagent samples in Ouchterlony plates.

3. The development of muscle during limb regeneration was tested by the agar diffusion method. Actomyosin and myosin can first be detected in the regenerate at the stage of hand formation ('palette' stage). Thereafter there is an upsurge of synthesis of these proteins during the 'early' and 'medium' digital stages. The appearance of these contractile proteins probably coincided with the time of myofibril formation.

4. A rise in nucleoprotein preceded the appearance of muscle protein and later decreased to a concentration characteristic of adult tissues. All tissues which were tested immunologically contained the nucleoprotein component.

5. Infusions of rabbit serum and anti-muscle serum into the regenerate yielded a cytotoxic response which varied according to the serum used, the reaction to the immune serum being more severe. There was a temporary cessation of regeneration for a few days, the duration of which was related to the severity of the reaction. Observations of the histopathology of infused regenerates revealed sarcolysis attributable to the specific effects of the anti-muscle serum.

6. Autoradiograms of limbs infused with I^{131} gamma globulin demonstrated the course and disposition of labelled protein within the regenerate and stump. There was localization of material about muscle-cells, suggesting a possible specificity of the reaction. The epidermis appeared to be an active organ for the elimination of foreign material.

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EXPLANATION OF PLATES

PLATE 1

FIGS. 1, 2, 3, 4. Reactions of rabbit antisera evoked by several different antigens tested against a single antigenic preparation in agar diffusion plates. Antisera against a given class of antigen from different rabbits are assigned successive letters (see below). Subsequent bleedings from the same rabbit are noted by a superscript; for example, anti-actomyosin A, B, C, and A^{I}, A^{II}, and A^{III}.

FIGS. 5, 6, 7. Agar diffusion plates with and without temperature artifacts. Extensive temperature striae are recorded in the accompanying Table with *. In figs. 5 & 6 the plates are duplicates except for temperature artifacts resulting from examination of the plate in fig. 6 during the course of the experiment.

		Reservoir contents						
Fig.	*Reaction type*	*Central*	*A*†	*B*	*C*	*D*	*E*	*F*
1		newt extract	anti-actomy. A	anti-my. A	anti-my. B	anti-actin A	anti-actin A^1	anti-actomy. B
	actomyosin		–	+	–	–	–	+
	myosin		–	–	+	–	–	
	non-specific		–	–			–	
2		newt extract	anti-actomy. A	anti-whole newt A	anti-whole newt A^I	anti-whole Newt B	anti-whole newt B^1	anti-whole newt C
	actomyosin		–	–	–	–	–	
	myosin		–	+	+	–	–	–
	non-specific		–	–	–	+	–	–
3		newt extract	anti-actomy. A	anti-whole newt D	anti-whole newt D^I	anti-muscle A	anti-whole newt E	anti-whole newt E^1
	actomyosin		–	–		–		
	myosin		–	–	–	trace	+	+
	non-specific		–	–	–	–	–	trace
4		newt extract	anti-actomy. A	anti-muscle B	anti-muscle B^I	anti-muscle B^{II}	anti-muscle B^{III}	anti-newt F
	actomyosin		–	–	–	+	+	+
	myosin			–	–	+	–	+
	non-specific			–	–	+	+	+
5		anti-actomy. A	newt extract	newt extract 50%	newt extract 25%	testis	testis 50%	dialysed newt extract
	actomyosin		–	+	+	–	–	+
	myosin		–	+	+	–	–	+
	non-specific		–	+	+	+	+	+
6	actomyosin		–*	+*	+	–	–	+
	myosin		–*	+*	+	–	–	+
	non-specific		–	+	+	+	+	+
7		anti-actomy. A	muscle	skin	early dig.	muscle	late bud	big medium bud
	muscle		+	–	–	–*		
	non-specific		+	–	+	+	–	

† Reservoir A is in the upper centre of each figure; the others are enumerated in clockwise order.

PLATE 2

The cross reactivity of several antigens and antisera demonstrated by agar diffusion

FIG. 8. Skeletal muscle extract tested with several antisera to demonstrate homologous reactions.

FIG. 9. The antisera shown in fig. 8 tested with a mixed organ extract. Only the non-specific (nucleoprotein) band is present.

FIGS. 10, 11, 12, 13. Reactions of extracts of regenerating limbs to anti-actomyosin and anti-myosin sera. Distinct bands indicating muscle proteins are evident in the digital stages. The first detectable reactions are in the palette stage.

		Reservoir contents							
Fig.	*Reaction type*	*Central*	*A*	*B*	*C*	*D*	*E*	*F*	*G*
8		muscle	anti-actomy. A	anti-actomy. A^I	anti-actomy.	anti-actomy.	anti-my.	anti-my.	anti-my.
	muscle		+	+	+	+	+	+	+
	non-specific		+	−	−	−	−	−	−
9		mixed extract of testis ovary spleen	anti-actomy. A	anti-actomy. A^I	anti-actomy.	anti-actomy.	anti-my.	anti-my.	anti-my.
	muscle		−	−	−	−	−	−	−
	non-specific		+	+	+		−	−	−
10		anti-actomy. A	newt my.	newt actomy.	frog my.	big early bud	medium bud	late bud	
	muscle		+	+	+	−	−	−	
	non-specific		trace		−	+	−	+	
11		anti-actomy. A	whole newt extract	early palette	very early palette	moderate digital 50% conc.	early digital	moderate digital	
	muscle		+	−	−	−	+	+	
	non-specific		+	+	+	+	+	+	
12		anti-actomy. A	whole newt extract	moderate digital	medium digital A	moderate digital	medium digital B	moderate digital B at 50%	
	muscle		+	+	+	+	+	+	
	non-specific		+	+	+	+	+	+	

Fig.	*Reaction type*	*Central*	*A*	*B*	*C*	*D*	*E*	*F*
13		anti-my. A absorbed with newt blood	whole newt extract	medium digital	very early digital	moderate digital 50% conc. as in F	early digital	moderate digital
	muscle		+	+	+	trace	+	+
	non-specific		—	—	—	—	—	—

PLATE 3

The macroscopic appearance of newt limbs after infusion of anti-muscle or control serum. All sera used were absorbed with small quantities of newt blood. The infusion time was three hours. The ventral aspect of each limb is pictured.

Normal serum and anti-serum produced cytotoxic reactions and delays in regeneration, the effects of the antisera being more severe.

FIG. 14. A big early regenerate about 8 hours after infusion with normal rabbit serum. Note the extensive blister over the entire limb. The stump also shows extensive hyperaemia.

FIG. 15. An advanced medium regenerate one day after infusion with anti-myosin serum. Opacity of the epidermis, haemorrhagic areas and hyperaemia are evident.

FIG. 16. A short medium palette regenerate, untreated control, 24 days after amputation.

FIG. 17. A regenerate delayed by the infusion of anti-myosin serum. The regenerate was infused when it and its control were in the early bud stage, 12 days after amputation. In the 12 days following infusion the experimental limb had not progressed beyond the advanced early stage.

FIG. 18. An untreated moderate digital regenerate 32 days after amputation: elbow and fingers are forming.

FIG. 19. The delay caused by infusion of anti-muscle serum is still evident 13 days after infusion. The infused limb is developing at a slower rate than is its control.

FIG. 20. Section of a normal untreated 10-day regenerate in the very early bud stage. Normal muscle fibre dedifferentiation is shown in the distal portion of the stump. Thickness 10 μ; stained with the Bodian silver method and counterstained with orange G, magnification about ×200.

FIG. 21. The effect of anti-myosin infusion upon the muscle of a palette regenerate 24 days after amputation. Muscle fibres, attached to tendon and therefore not sectioned on amputation, are seen in various abnormal states. Upper right: the sarcoplasm is present but amorphous. Lower right: empty sarcolemmal sheaths. Dark droplets and debris can be seen amongst damaged muscle fibres. Thickness 10 μ, stained with the Bodian silver method and counterstained with orange G. ×600.

FIG. 22. A 22-day-old early digital stage regenerate infused with anti-actomyosin serum for three hours at 19 days after amputation. All tissues other than muscle appear healthy; mitotic figures are found but muscle fibres are disintegrating. The level of the photograph is below the original amputation. Centre: note that a small fragment of a cross-striated fibre remains near amorphous mass of other young muscle-cells. Thickness 10 μ, stained with the Bodian silver method and counterstained with orange G. ×600.

FIG. 23. Section of a regenerating limb fixed shortly after infusion of I^{131}-labelled gamma globulin. This advanced early regenerate was infused 16 days after amputation. The label is restricted predominantly to the blastema. Material can be seen to be dispersing from the infusion site through the skin, blood-vessels, following fascial planes, and within tissue spaces. The infusion needle path is visible in this section. The section is 10 μ thick, stained with acid haematoxylin. ×20.

FIG. 24. Section of a limb fixed 9 hours after infusion of I^{131}-labelled gamma globulin. The label is more concentrated in muscle than in other tissues, e.g. at the lower left of photograph. Most activity has been removed from the tissue spaces. A mixture of blastema and degenerating muscle-cells which are well labelled can be seen at the upper left region of the photograph. Thickness 10 μ, stained with acid haematoxylin. ×80.

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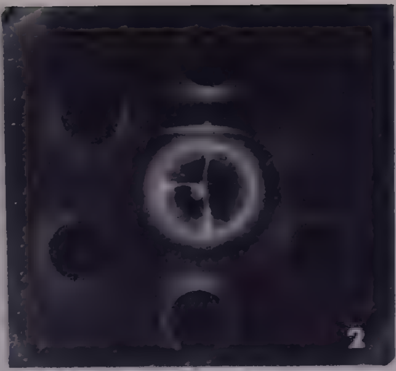
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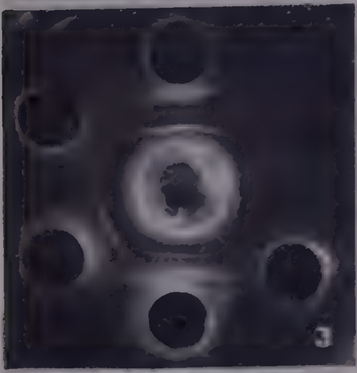
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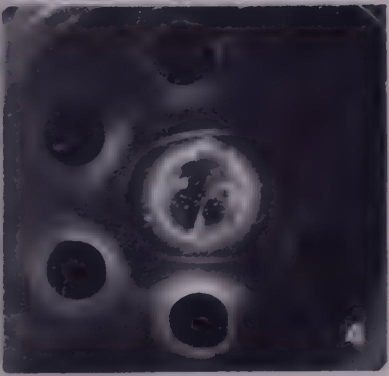
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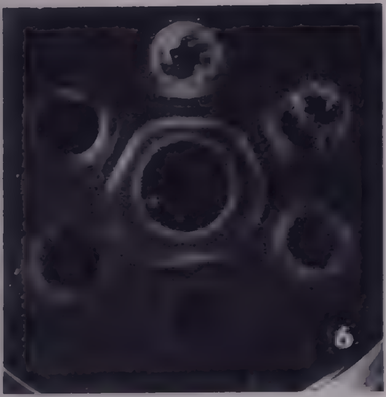
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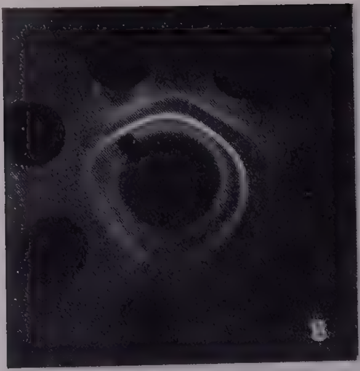
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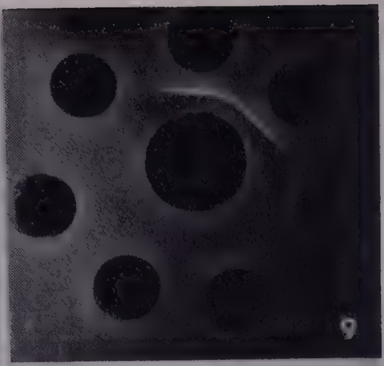
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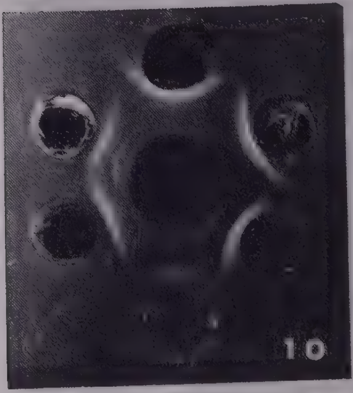
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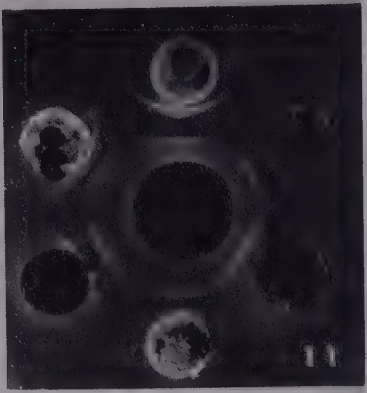
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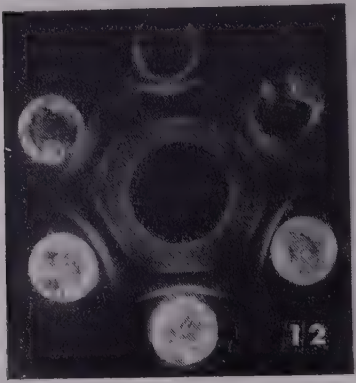
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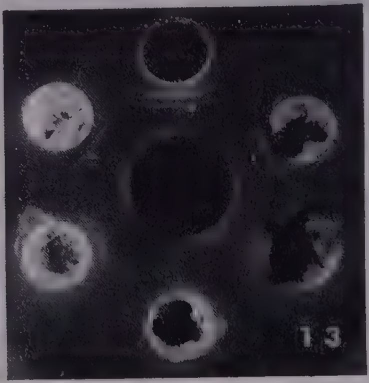
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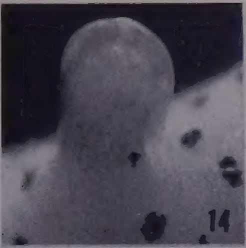
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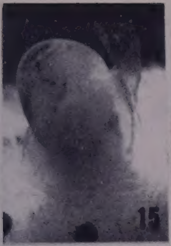
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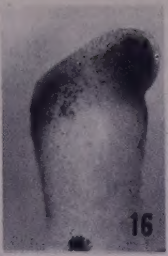
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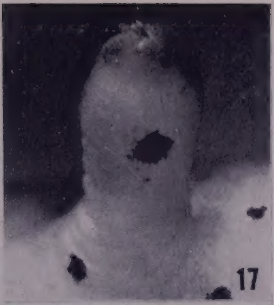
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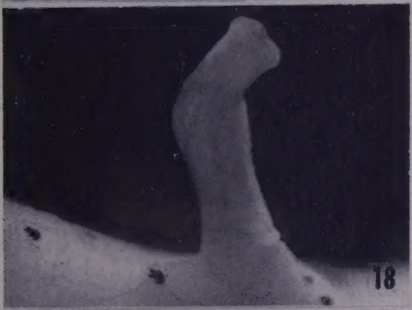
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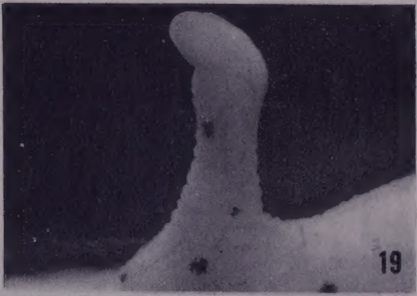
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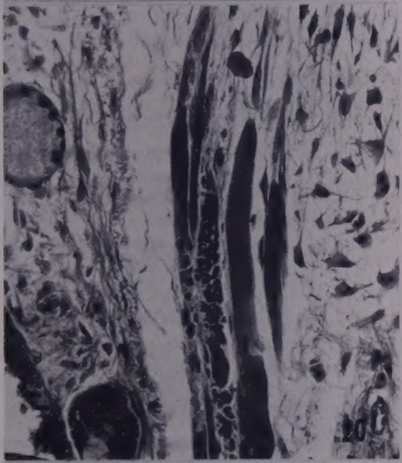
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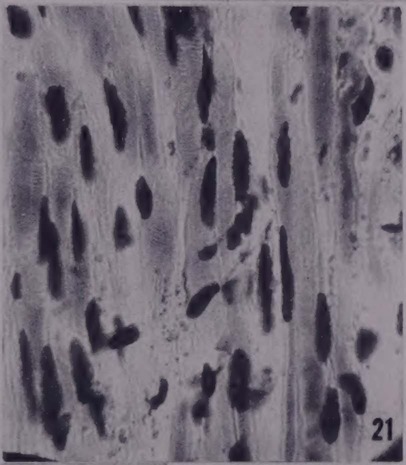
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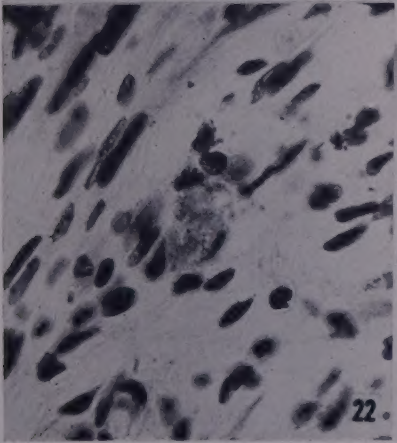
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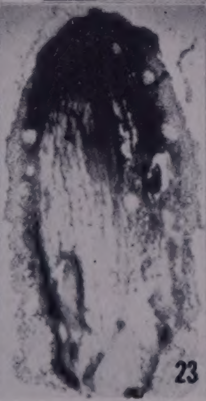
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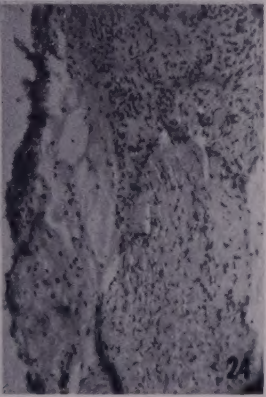
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Plate 3

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